DANUSA GEBIN DAS NEVES

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Dr. Márcio Gilberto Zangeronimo
Orientador

Dr. Édison José Fassani
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Coorientadores

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APROVADA em 28 de junho de 2019

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“Feliz aquele que transfere o que sabe e aprende o que ensina.
O saber se aprende com mestres e livros.
A Sabedoria, com o corriqueiro, com a vida e com os humildes.
O que importa na vida não é o ponto de partida, mas a caminhada.
Caminhando e semeando, sempre se terá o que colher”

Cora Coralina

RESUMO

Os efeitos da injeção *in ovo* de glicerol (GLY) e do fator de crescimento semelhante à insulina (IGF-I) sobre a eclodibilidade, parâmetros bioquímicos, morfometria intestinal, desempenho e características de carcaça de frangos de corte foram avaliados. Para isso, três experimentos foram conduzidos; o primeiro avaliou diferentes concentrações de GLY (controle não-injetado e cinco soluções salinas contendo 0, 10, 20, 40 e 80 nmol/mL de GLY); o segundo diferentes doses de IGF-I (controle não-injetado e cinco soluções salinas contendo 0; 12,5; 25; 37,5 e 50 ng/embrião de IGF-I); e o terceiro a associação de GLY e IGF-I (controle não-injetado e quatro soluções salinas contendo 0, 10 nmol/mL de GLY, 100 ng/mL de IGF-I e GLY + IGF-I). As injeções foram realizadas aos 17 dias de incubação. No primeiro experimento, as doses de 10, 20 ou 40 nmol/mL de GLY reduziram (P<0,01) o tempo de incubação e as doses de 20, 40 ou 80 nmol/mL de GLY diminuíram (P<0,01) a glicemia. A dose de 80 nmol/mL de GLY diminuiu (P=0,01) o glicogênio muscular e as doses de 40 ou 80 nmol/mL de GLY aumentaram (P=0,02) a atividade da frutose 1,6-bisfosfatase (FBP). O uso de 20 ou 40 nmol/mL de GLY aumentou (P<0,05) o peso do duodeno no 1º e no 7º dia de idade. As diferentes concentrações de GLY aumentaram (P=0,02) o peso de peito aos 7 dias de idade e a deposição de gordura abdominal aos 40 dias de idade. Aos 7, 14 e 21 dias de idade, as doses de 10 ou 20 nmol/mL de GLY resultaram em frangos com melhor (P<0,05) desempenho. Nenhum efeito de GLY foi observado (P>0,05) sobre a eclodibilidade, atividade da glicerol quinase, teor de glicogênio hepático, morfometria intestinal e ácido úrico. No segundo experimento, a dose de 50 ng/embrião de IGF-I aumentou (P<0,05) a eclodibilidade e o glicogênio muscular. Menor (P<0,01) glicemia e maior (P<0,01) atividade da FBP foram observadas com a injeção de 12,5 ng ou mais de IGF-I/embrião. Aos 7 dias de idade, as doses de 25, 37,5 ou 50 ng/embrião de IGF-I aumentaram (P=0,04) o peso de peito. Maior (P<0,01) relação altura de vilos/profundidade de cripta no duodeno foi observada com injeção de 12,5, 25 ou 37,5 ng/embrião de IGF-I após a eclosão e aos 7 dias de idade com as doses de 37,5 ou 50 ng/embrião de IGF-I no jejuno e no íleo com as diferentes concentrações de IGF-I. Nenhum efeito de IGF-I foi observado (P>0,05) sobre o tempo de incubação, ácido úrico, desempenho e características de carcaça. No terceiro experimento, a injeção de GLY ou de

IGF-I aumentou (P=0,01) o teor de glicogênio hepático. Maior (P<0,05) teor de glicogênio muscular e altura de vilos no jejuno e no íleo após a eclosão foi obtido com a injeção de GLY. As substâncias aumentaram (P=0,05) o peso de fígado e diminuíram (P=0,05) a atividade da FBP. Aos 7 dias de idade, menor (P<0,01) peso de fígado e maior (P=0,02) peso de íleo foram observados, respectivamente, com GLY e IGF-I. Aos 14 dias de idade, a injeção de IGF-I resultou em frangos de corte com maior (P<0,01) ganho de peso e melhor (P=0,05) conversão alimentar. Aos 35 e 42 dias de idade, o uso de GLY ou IGF-I ou GLY + IGF-I aumentou (P<0,01) o consumo de ração e o ganho de peso em relação ao grupo não injetado. Nenhum efeito das substâncias injetadas foi observado (P>0,05) sobre a eclodibilidade, tempo de eclosão, glicemia e características de carcaça aos 42 dias de idade. Conclui-se que o GLY associado ou não ao IGF-I pode ser utilizado como substrato na nutrição *in ovo* de frangos de corte para melhorar o desempenho dos frangos de corte e que o IGF-I pode ser utilizado na dose de 50 ng/embrião para aumentar a eclodibilidade.

Palavras-chave: Avicultura**.** Desempenho Pós-eclosão. Desenvolvimento Embrionário. Epitélio Intestinal. Metabolismo. Substrato Energético. Fator de Crescimento. Eclodibilidade.

ABSTRACT

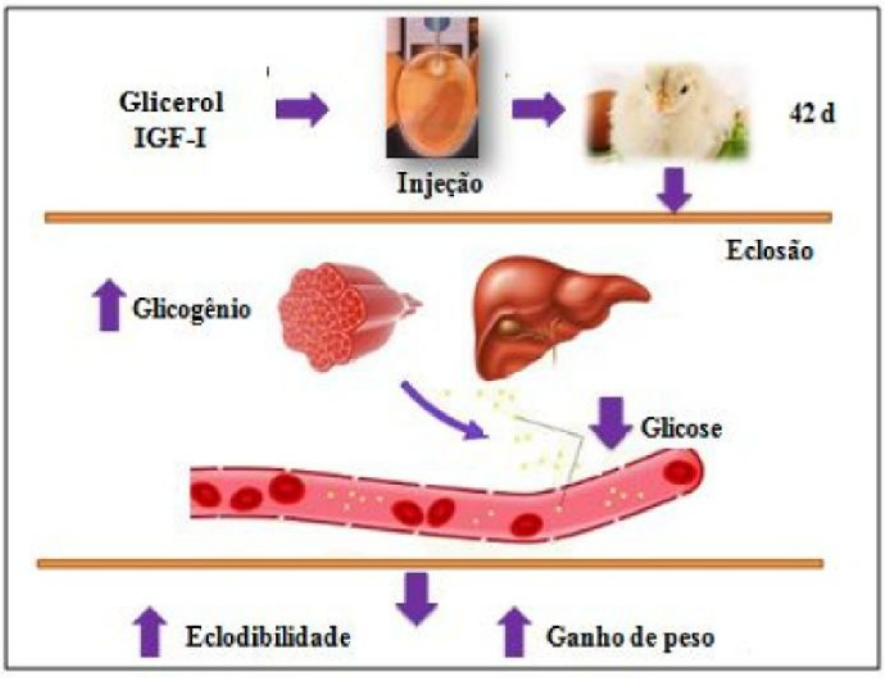
This study was conducted to determine the effects of *in ovo* feeding of glycerol (GLY) and insulin-like growth factor (IGF-I) on hatchability, biochemical parameters, intestinal morphometry, performance and carcass characteristics of broilers. For this three experiments were conducted; the first evaluated different concentrations of GLY (non-injected control and five saline solutions containing 0, 10, 20, 40 and 80 nmol/mL GLY); the second different doses of IGF-I (non-injected control and five saline solutions containing 0, 12.5, 25, 37.5 and 50 ng/IGF-I embryo); and the third the association of GLY and IGF-I (non-injected control and four saline solutions containing 0, 10 nmol/mL GLY, 100 ng/mL IGF-I and GLY + IGF-I). Injections were performed at 17 days of incubation. In the first experiment, doses of 10, 20 or 40 nmol/mL GLY reduced ($P<0.01$) the incubation time and doses of 20, 40 or 80 nmol/mL GLY decreased ($P<0.01$) the glycemia. The dose of 80 nmol/mL GLY decreased ($P=0.01$) muscle glycogen and the doses of 40 or 80 nmol/mL GLY increased ($P=0.02$) the fructose 1,6-bisphosphatase (FBP) activity. The use of 20 or 40 nmol/mL GLY increased ($P<0.05$) the duodenum weight in the 1st and 7th days of age. The different concentrations of GLY increased ($P=0.02$) the breast weight at 7 days of age and the deposition of abdominal fat at 40 days of age. At 7, 14 and 21 days of age, doses 10 or 20 nmol/mL GLY resulted in broilers with better ($P<0.05$) performance. No effect of GLY was observed ($P>0.05$) on hatchability, glycerol kinase activity, hepatic glycogen content, intestinal morphometry and uric acid. In the second experiment, the dose of 50 ng/embryo IGF-I increased ($P<0.05$) the hatchability and muscle glycogen. Lower ($P<0.01$) glycemia and higher ($P<0.01$) FBP activity were observed with the injection of 12.5 ng or more of IGF-I/embryo. At 7 days of age, doses of 25, 37.5 or 50 ng/embryo IGF-I increased ($P=0.04$) the breast weight. The highest ($P<0.01$) villus height/crypt depth ratio in the duodenum was observed with injection of 12.5, 25 or 37.5 ng/embryo IGF-I after hatching and at 7 days of age with doses of 37.5 or 50 ng/embryo IGF-I in the jejunum and in the ileum with the different concentrations of IGF-I. No effect was observed ($P>0.05$) on incubation time, blood uric acid, performance or carcass characteristics. In the third experiment, the injections of GLY or IGF-I increased ($P=0.01$) the hepatic glycogen. Higher ($P<0.05$) muscle glycogen content and height villus in the jejunum and in the ileum after hatching were obtained with injection of GLY. The substances increased ($P=0.05$) the liver weight and decreased ($P=0.05$) FBP activity. At 7 days of age, lower ($P<0.01$) liver weight and greater ($P=0.02$) ileum weight were observed, respectively, with GLY and IGF-I. At 14 days of age, IGF-I resulted in broiler with higher ($P<0.01$) weight

gain and better (P=0.05) feed conversion. At 35 and 42 days of age, the use of GLY or IGF-I or GLY + IGF-I increased (P<0.01) feed intake and weight gain in relation to the non-injected group. No effects of the injected substances were observed (P>0.05) on hatchability, hatching time, glycemia and carcass characteristics at 42 days of age. It is concluded that GLY associated or not to IGF-I can be used as a substrate in *in ovo* nutrition to improve the performance of broiler chickens and that IGF-I can be used at dose of 50 ng/embryo to increase hatchability.

Keywords: Poultry. Post-hatch Performace. Embryo Development. Intestinal Epithelial. Metabolism, Energy Substrate. Growth Factor. Eclodibility.

RESUMO INTERPRETATIVO E RESUMO GRÁFICO

A técnica de alimentação *in ovo* estabeleceu uma nova estratégia na nutrição precoce e tem sido indicada para melhorar a eficiência da produção de frangos de corte. No entanto, o principal problema desse método é a redução da eclodibilidade. O objetivo desse estudo foi avaliar os efeitos da injeção de soluções contendo glicerol associadas ou não ao IGF-I sobre a eclodibilidade, parâmetros bioquímicos, morfometria intestinal, desempenho e características de carcaça de frangos de corte. No geral, observamos que a alimentação *in ovo* com IGF-I aumentou a eclodibilidade e a de glicerol associada ou não ao IGF-I melhorou alguns aspectos metabólicos de frangos de corte para incubação, o que refletiu na melhora do desempenho dos frangos de corte. Os resultados positivos observados no presente estudo sugerem que a injeção *in ovo* dessas substâncias pode ser considerada uma técnica viável a ser aplicada em incubatórios comerciais. Além disso, esses achados representam uma base para estudos futuros em relação ao uso de outras concentrações e ao tempo ideal para a realização da técnica.



Aumento da eclodibilidade e do ganho de peso em frangos de corte provenientes de ovos injetados com soluções contendo glicerol e/ou IGF-I.

SUMÁRIO

PRIMEIRA PARTE – REFERENCIAL TEÓRICO

1. INTRODUÇÃO

O elevado potencial genético da ave moderna para maior taxa de crescimento e aumento muscular tem alterado os requerimentos nutricionais dos embriões. O desequilíbrio entre a exigência embrionária para energia e aminoácidos e os nutrientes armazenados no ovo, especialmente a glicose, podem ser insuficientes e limitar o crescimento e o desenvolvimento das aves antes e após a eclosão. Alimentar o embrião durante a incubação através da injeção de nutrientes *in ovo* é uma forma de amenizar esse desequilíbrio e pode ser realizada através da vacinação *in ovo,* uma ferramenta que está se tornando cada vez mais popular nos incubatórios.

Momentos antes da eclosão, a alta demanda energética do processo de eclosão e a baixa oferta de O_2 dentro do ovo favorecem o metabolismo anaeróbico. Nesse momento, a manutenção da glicemia é dependente da quantidade de glicose contida nas reservas de glicogênio e da gliconeogênese a partir de glicerol, lactato e aminoácidos. Como consequência da redução da disponibilidade de aminoácidos para a síntese muscular, o crescimento e o desenvolvimento de pintos após a eclosão podem ser comprometidos. Assim, a aplicação da técnica de alimentação *in ovo* com a injeção de substâncias capazes de complementar às exigências nutricionais durante esse período poderia economizar o uso de aminoácidos, melhorar a eclodibilidade e o desempenho das aves até a idade de abate. Além disso, sabe-se que o número de fibras musculares é determinado durante o período embrionário e que os fatores de crescimento afetam o crescimento muscular. Supostamente, uma forma de otimizar o desenvolvimento muscular seria aumentar a disponibilidade de fatores de crescimento relacionados ao desenvolvimento das fibras por meio de injeção *in ovo* durante o desenvolvimento do embrião.

Está bem documentado que o glicerol é um importante precursor da gliconeogênese e que o fator de crescimento semelhante à insulina I (IGF-I) desempenha um papel importante no controle metabólico, diferenciação e crescimento tecidual. Acredita-se que o uso *in ovo* de glicerol e IGF-I possam aumentar a síntese de glicogênio e de proteínas, influenciar o metabolismo das aves na eclosão, promover melhorias no epitélio intestinal e favorecer o desenvolvimento após a eclosão, melhorando o desempenho e as características de carcaça de

frangos de corte até a idade de abate. Assim, o objetivo desta revisão foi retratar o potencial do glicerol e do IGF-I como possíveis substâncias a serem utilizadas na nutrição *in ovo*.

2. REVISÃO DE LITERATURA

2.1. Utilização de nutrientes pelo embrião

O desenvolvimento embrionário das aves é restringido pelo conteúdo de nutrientes presentes no ovo que, por sua vez, depende do peso do ovo, da linhagem genética e da idade da matriz (Vieira e Moran, 1998). Sendo assim, a composição nutricional do ovo fértil pode influenciar a utilização de nutrientes pelo embrião, a qualidade e a sobrevivência do pinto após a eclosão (Uni *et al.*, 2012; Retes *et al.*, 2018).

O ovo é constituído pela casca, albúmen e gema. A casca, além de ser uma barreira física contra a invasão bacteriana e perda excessiva de água, permite a troca de gases e disponibiliza cálcio para formação e calcificação dos ossos nos estágios mais avançados da embriogênese (Alcroft, 1964). Além do cálcio, outros minerais também são fornecidos pela casca como magnésio, fósforo (Romanoff e Romanoff, 1949), cobre, zinco, manganês e ferro (Richards, 1989).

O albúmen, além de proteger o embrião das infecções microbianas e manter a inércia térmica do ovo, fornece água, aminoácidos e minerais (Sturkie, 1998). Sua estrutura é composta por quatro camadas proteicas distintas, de diferentes graus de viscosidade e turbidez (Romanoff e Romanoff, 1949). Dentre as principais proteínas encontradas no albúmen tem-se a ovoalbumina, a ovotransferrina ou conalbumina, a ovomucóide, as ovoglobulinas (G2 e G3), a lisozima e as ovomucinas α e β (Mueller *et al.*, 2015). A ovoalbumina é fonte de aminoácidos para o embrião e, devido sua homologia com a família serpin de inibidores de proteases, suprime a atividade enzimática no ovo. A ovotransferrina age como queladora de ferro prevenindo o crescimento bacteriano dentro do ovo. A ovomucóide é inibidora de proteases, principalmente de tripsina, a qual evita a degradação da albumina do ovo. Entre as ovoglobulinas têm-se as flavoproteínas; as ovomacroglobulinas, a ovoinibidor e a cistatina que atuam como inibidores de proteases; as proteínas transportadoras de riboflavina, ferro, zinco e cobre; e a avidina que possui a capacidade de quelatar minerais e inibir o crescimento bacteriano. Além disso, a lisozima tem atividade lítica contra paredes de células bacterianas gram-negativas. E, por fim, a α-ovomucina (glicoproteína) e a β-ovomucina (60%

carboidratos) proteínas fibrosas insolúveis responsáveis pelo gel do albúmen que bloqueiam a invasão de microrganismos e podem expressar propriedades antivirais (Mueller *et al*., 2015).

A gema representa uma importante fonte de proteínas e lipídeos para o embrião (Gonçalves *et al.*, 2013). Sob o ponto de vista nutricional, a gema é a fração mais rica do ovo. As proteínas e os lipídeos apresentam-se majoritariamente na forma de lipoproteínas de densidade muito baixa (VLDLs). Os lipídios presentes na gema correspondem 65% de triglicerídios, 23,3% de fosfolipídios e 5,2% de colesterol (Privett *et al.*, 1962). Os fosfolipídios predominantes são a fosfatidilcolina e a fosfatidiletanolamina. Os principais ácidos graxos componentes da gema são o palmítico e o esteárico. Em relação às proteínas, a fosvitina e a lipovitelina estão presentes na gema granular, enquanto que as livetinas são as componentes do plasma da gema e podem ser divididas em α, β e γ-livetinas. As α-livetinas são albuminas séricas, as β-livetinas são glicoproteínas e as γ-livetinas são γ-globulinas que correspondem às imoglobulinas G, comumente chamadas imunoglobulinas da gema ou IgY (Leslie e Clem, 1969).

Ao considerar um ovo fértil de 60 g de peso com cerca de 6 g de casca, 34 g de albúmen e 20 g de gema, supõe-se que o embrião utiliza para o seu desenvolvimento aproximadamente 40 g de água, 7 g de proteína, 6 g de lipídeo e 425 mg de carboidrato (Freeman e Vince, 1974). Desse último, apenas 0,3% encontram-se na forma de glicose livre (Romanoff e Romanoff, 1949). Dessa forma, o metabolismo embrionário é voltado para o metabolismo de aminoácidos e lipídeos para a síntese protéica e produção de energia com as vias metabólicas dependentes da disponibilidade de O_2 (Hu, 2013).

No início do desenvolvimento embrionário, o acesso ao O_2 é limitado devido à imaturidade das células sanguíneas e ao reduzido desenvolvimento do sistema vascular. A energia gasta nesse momento decorre de grande parte da glicólise, através de glicose prontamente acessível ou por um aumento transitório de lactato que ocorre até a membrana corioalantóide, membrana que circunda o embrião, se tornar funcional (Cirotto e Arangi, 1989).

À medida que a membrana corioalantoide inicia sua função respiratória assegurando a troca de O_2 e CO_2, via poros da casca, o sistema vascular se encontra totalmente desenvolvido (Levinsohn *et al.*, 1984). A partir desse momento, os ácidos graxos são utilizados como fonte primária de energia e como base para o desenvolvimento embrionário (Moran, 2007). Assim, a formação de adenosina trifosfato impulsiona os processos metabólicos, enquanto o calor e a água resultantes da oxidação ajudam a incubação (Whittow e Tarazawa, 1991). Além disso,

devido o baixo coeficiente respiratório (RQ = 0,07), os carboidratos são conservados sem que haja o acúmulo de corpos cetônicos (Moran, 2007; Barbosa, 2011).

Entre o 10º e 12º dia de incubação, a expansão do embrião dentro do saco amniótico associado ao aumento de seus movimentos, faz com que parte do albúmen e o âmnio formem uma conexão sero-amniótica na parte mais fina do ovo (Barbosa, 2011). Dessa forma, o embrião ingere essa mistura de albúmen e fluido amniótico que é absorvida através do sistema gastrointestinal (Sugimoto *et al.*, 1999), ocorrendo absorção parcial de proteínas inteiras pelos enterócitos durante a passagem pelo duodeno e jejuno. Esses nutrientes são utilizados na maturação dos órgãos viscerais e armazenados na forma de glicogênio no músculo e no fígado, os quais serão utilizados no processo de eclosão. Esse consumo e absorção tornam-se contínuos até que a mistura albúmen-âmnio desapareça e a bicagem interna se inicie (Moran, 2007).

Com o consumo oral, juntamente com a inibição da digestão por fatores anti-tripsina, embora a presença das enzimas pancreáticas (Yoshizaki *et al.*, 2002), as proteínas do albúmen e a IgA são evidenciadas no sangue e continuam a ser detectáveis após a eclosão (Barbosa, 2011). A IgA, presente apenas no albúmen proporciona proteção a superfície da mucosa intestinal com concentração máxima no 19º dia de incubação. Essas proteínas do albúmen citadas anteriormente são glicoproteínas ou proteínas glicadas, ou seja, possui carboidratos ligados a proteína, os quais são utilizados pelo embrião durante a gliconeogênese com o objetivo de preservar os aminoácidos para a síntese protéica (Muramatsu *et al.*, 1990). Como resultado, a glicose no sangue aumenta para manter a deposição de glicogênio hepático e muscular (Moran, 2007).

Ao mesmo tempo em que parte do albúmen está fluindo para dentro da cavidade amniótica a outra parte do albúmen juntamente com as enzimas pancreáticas entram no saco vitelínico através da haste com o auxílio da atividade antiperistáltica do cólon (Sugimoto *et al.*, 1999). Células do saco vitelínico, mediados por receptores, absorvem as VLDLs intactas por mecanismo de endocitose (Noble *et al.*, 1988). Essas células possuem um conjunto diverso de enzimas capazes de alterar os lipídeos absorvidos libertando-os para a circulação (Powell *et al.*, 2004). Além da endocitose mediada pelos receptores de lipoproteínas da gema para as células endoteliais da membrana do saco vitelínico, outros nutrientes (glicose e minerais) são carreados para os vasos sanguíneos atingindo os tecidos embrionários (Yadgary e Uni, 2012).

Concomitantemente, o aumento de CO_2 em conjunto com a anidrase carbônica cria condições ácidas para a dissolução de botões mamários adjacentes à interface de membrana de corioalantoíde-casca e ocorre a agregação dos grânulos de cálcio na circulação, os quais permanecem no saco vitelino até a eclosão, favorecendo a calcificação do esqueleto (Moran, 2007).

Durante a última semana de incubação, a beta oxidação dos ácidos graxos da gema é a principal fonte de energia para o embrião (Speake *et al.*, 1998). No entanto, devido ao aumento da demanda energética pelo processo de eclosão e a relativa baixa quantidade de oxigênio disponível durante os últimos dois ou três dias de incubação, os ácidos graxos não são capazes de fornecer toda a energia necessária ao embrião (Moran, 2007). O fornecimento limitado de O_2 pouco antes da eclosão até a bicagem externa da membrana faz com que a atividade do ciclo de Krebs seja drasticamente reduzida (De Oliveira *et al.*, 2008). Sendo assim, nesta fase, a estratégia metabólica muda e o embrião passa a realizar catabolismo anaeróbico de glicose. A produção de glicose, a partir de substratos ricos em carbono como glicogênio, aminoácidos, glicerol e outros carboidratos, por meio da gliconeogênese torna-se essencial nos últimos dias de incubação e durante a eclosão (Moran, 2007). Em consequência da bicagem da membrana interna, a função pulmonar começa a fornecer a quantidade de oxigênio adequada para o catabolismo de ácidos graxos, a atividade do ciclo de Krebs aumenta novamente e os lipídios continuam como principal fonte de energia para o embrião (De Oliveira *et al.*, 2008).

Em suma, a demanda de energia eleva-se com o crescimento do embrião (Moran, 2007). Portanto, a composição nutricional do ovo fértil pode influenciar o desenvolvimento embrionário e qualquer interferência durante ess glicosadas e período, especialmente no final da incubação, pode afetar significativamente a sobrevivência dos embriões e o desempenho das aves após a eclosão. Na tentativa de aumentar a eclodibilidade e melhorar o desenvolvimento após eclosão, o fornecimento de nutrientes *in ovo* durante o período de incubação tem sido testado (Uni *et al.*, 2005; Yu *et al.*, 2018a; Zhao *et al.*, 2018).

2.2. A nutrição *in ovo* como alternativa para melhorar o desenvolvimento antes e após a eclosão

A seleção intensiva de frangos de corte para maior taxa de crescimento e aumento muscular tem alterado as necessidades nutricionais dos embriões (Nasir e Peebles, 2018). O

desequilíbrio entre a exigência embrionária para energia e aminoácidos e os nutrientes armazenados no ovo pode limitar o crescimento e o desenvolvimento das aves antes e após a eclosão (Retes *et al.*, 2018). Além disso, o *déficit* de energia no período que antecede à eclosão esta relacionada à mortalidade embrionária e, como conseqüência redução do número de ovos eclodidos (Moreira Filho *et al.*, 2018)

Uma forma de amenizar esse desequilíbrio é alimentar o embrião durante a incubação através da injeção *in ovo* de nutrientes. A injeção, geralmente, é realizada no âmnio (Nasir e Peebles, 2018) com o objetivo de suplementar o líquido amniótico que será consumido pelo embrião antes da eclosão (Uni e Ferket, 2004). Além disso, o âmnio é o local ideal para a realização da vacinação *in ovo* (Peebles, 2018). Como a vacinação *in ovo* está se tornando uma ferramenta popular nos incubatórios, existe a possibilidade de nutrir o embrião nesse período.

O pinto de um dia de idade de qualidade é o elo crucial entre o incubatório e a granja de frangos de corte (Jha *et al.*, 2019). Na prática, as aves que eclodem de forma precoce permanecem dentro da incubadora sem acesso à alimentação e à água, dependendo apenas dos nutrientes contidos no resíduo vitelínico. Além disso, manejos como a determinação de sexo, classificação das aves, vacinação e transporte até as granjas prolongam ainda mais o período de restrição, algumas vezes por até 72 horas (Willemsen *et al.*, 2010; Kornasio *et al.*, 2011). Durante esse tempo de restrição alimentar e hídrica, as aves chegam a perder cerca de 4 g de peso corporal a cada 24 horas, decorrente das perdas de umidade bem como a utilização de nutrientes contidos no resíduo vitelínico (Noy e Sklan, 1998; Batal e Parsons, 2002). Esse atraso ocasiona danos irreversíveis, tais como retardamento do peso corporal, redução do desenvolvimento muscular (Kornasio *et al.*, 2011; Lamot *et al.*, 2014) e limitação do trato gastrointestinal (Uni *et al.*, 1998; Halevy *et al.*, 2000; Adeleye *et al.*, 2018).

A limitação do trato gastrointestinal, por sua vez, prejudica a absorção de nutrientes da ração e torna os pintos mais susceptíveis aos agentes patogênicos que prejudicam a saúde intestinal (Dibner *et al.*, 1998). Assim, é desejável que o trato gastrointestinal se torne fisiologicamente maduro o mais cedo possível, ou seja, quanto mais rápido o intestino alcançar sua capacidade funcional, mais cedo a ave poderá utilizar os nutrientes da dieta, crescer de acordo com seu potencial genético e ser resistente aos processos infecciosos (Uni *et al.*, 2003), evitando o comprometimento da homogeneidade do lote, do peso de abate e do rendimento dos cortes.

Uma das formas de estimular o desenvolvimento precoce do trato gastrointestinal é garantir a presença de nutrientes altamente digestíveis antes e imediatamente após a eclosão (Noy e Uni, 2010). Isso tem sido obtido pela nutrição *in ovo* (Moreira Filho *et al.*, 2018; Tasharofi *et al.*, 2018). Um dos primeiros trabalhos publicados em nutrição *in ovo* foi realizado por Al-Murrani (1982), o qual inoculou no saco vitelínico de ovos embrionados de frangos de corte, aos 7 dias de desenvolvimento, uma mistura de aminoácidos idêntica à composição encontrada no ovo. Nesse trabalho, foi obtido maior peso ao nascimento e aos 56 dias de idade das aves. Porém, sua intenção não era fazer nutrição *in ovo*, mas provar que galinhas poedeiras precisavam de proteína adicional em suas dietas. Posteriormente, a maioria dos estudos realizados tinha como objetivo a vacinação *in ovo*, prática que se tornou amplamente adotada pela indústria avícola (Johnston *et al.*, 1997) e é considerada uma das maiores contribuições da pesquisa com aves (Smith, 2006). Somente em 2003 as pesquisas sobre nutrição *in ovo* foram retomadas (Uni e Ferket, 2004). Desde então, diversos estudos têm mostrado que embriões são capazes de utilizar soluções nutritivas injetadas *in ovo* obtendo melhora no desenvolvimento no estágio final de incubação e garantindo o fornecimento de nutrientes antes e após a eclosão (Yu *et al.*, 2018b). A injeção de soluções nutritivas podem ainda aumentar o desenvolvimento muscular (Zhang *et al.*, 2017), a eficiência alimentar (Araujo *et al.*, 2018), melhorar a mineralização óssea (Abbasi *et al.*, 2017), fortalecer o sistema imunológico (Bhanja *et al.*, 2014; Goel *et al.*, 2017) e estimular o desenvolvimento das células envolvidas no processo digestivo (Berrocoso *et al.*, 2017; Gao *et al.*, 2017).

Como visto, nutrição *in ovo* é um conceito aceitável com vantagens claramente reconhecidas. No entanto, a grande questão continua a ser qual a melhor forma de aplicar essa técnica, bem como qual substâncias a serem utilizadas.

2.3. O potencial do glicerol como substância a ser utilizada na nutrição *in ovo*

O glicerol ou propano-1,2,3-triol é um composto orgânico pertencente à função álcool, que se apresenta como líquido à temperatura ambiente (25 ºC), higroscópico, inodoro, viscoso e de sabor adocicado (Iupac, 1993). Esse composto pode ser proveniente das gorduras animais, dos óleos vegetais ou da indústria petroquímica (Shuchardt *et al.*, 1998). No organismo, a disponibilidade do glicerol é derivada da lipólise das lipoproteínas, glicerolipídeos e triglicerídeos ou a partir da gordura dietética (Hagopian *et al.*, 2008).

O terço final do desenvolvimento embrionário é um período crítico e dinâmico, devido ao aumento da taxa de crescimento e a preparação do embrião para a eclosão. Nesse momento, embora a quantidade de glicose seja pequena (0,3% na forma livre) no ovo fértil, esse carboidrato é essencial para o desenvolvimento, sobrevivência e eclosão das aves. Portanto, o metabolismo embrionário poupa a utilização de glicose, aumenta a sua concentração no sangue e o seu armazemanento na forma de glicogênio com o decorrer do desenvolvimento embrionário (Sunny e Bequette, 2010).

O aumento de glicose na corrente sanguínea e dos depósitos de glicogênio muscular e hepático ocorre a partir do 13º dia de incubação pela gliconeogênese e glicogênese (Picardo e Dickson, 1982). Os estoques de glicogênio no fígado e nos músculos são essenciais para o rompimento da casca pelo embrião e sua eclosão, bem como para a sobrevivência do pinto até que eles tenham acesso à água e alimentação. Além disso, a quantidade de glicogênio têm sido correlacionada positivamente ao peso à eclosão (Christensen *et al.*, 2000), pois se exauridas essas reservas, o embrião começa a degradar proteína muscular para geração de energia (Dal Pont *et al.*, 2019).

O fornecimento limitado de O2, pouco antes da eclosão até a bicagem da membrana interna, reduz a atividade do ciclo de Krebs (De Oliveira *et al.*, 2008). Logo, a produção de glicose a partir de substratos ricos em carbono como aminoácidos, glicerol e lactato, por meio da gliconeogênese, torna-se ainda mais essencial nos últimos dias de incubação e durante a eclosão (Moran, 2007). Entretanto, mesmo que vários substratos possam servir como precursores para a síntese de glicose e glicogênio, sua preferência varia com a disponibilidade, estágio de desenvolvimento embrionário e localização de enzimas (Sunny, 2008). Estudos avaliando os efeitos da injeção *in ovo* de glicose (Leitão *et al.*, 2008) e de ácidos graxos (Pedroso *et al.*, 2006) no final do desenvolvimento embrionário observaram redução da eclodibilidade e falta de melhorias no desempenho das aves após a eclosão. Já o estudo de Sunny e Bequette (2011), avaliando a contribuição do glutamato, glutamina e glicerol para a síntese de glicose, glicogênio e aminoácidos não essenciais durante o desenvolvimento embrionário, concluiu que o glicerol é o principal substrato precursor para a síntese dos mesmos, além de ser um importante substrato anaplerótico para o ciclo de Krebs dos embriões. A utilização do glicerol como substrato para esses compostos é ainda mais significativa no terço final da incubação (19º dia do desenvolvimento embrionário) (Hu *et al.*, 2017). Além de poupar o uso de aminoácidos para formação de glicose, estudos relatam que o

glicerol é capaz de inibir a atividade das enzimas fosfoenolpiruvato carboxiquinase e glutamato desidrogenase, favorecendo a deposição de proteína corporal (Cerrate *et al.*, 2006).

Devido à sua função gliconeogênica e poupadora de aminoácidos, proporcionando aumento de glicose e reservas de glicogênio, evitando o catabolismo muscular antes e após a eclosão, o glicerol tem se mostrado interessante composto para ser utilizado na nutrição *in ovo*. Além disso, o aumento da atividade da glicerol quinase após a injeção de glicerol demonstrou a capacidade dos embriões em utilizá-lo (Neves *et al.*, 2016). Apesar de não ter aumentado o peso dos pintos após a eclosão (Neves *et al.*, 2016; Dal Pont *et al.*, 2019), a injeção de glicerol promoveu melhorias no desempenho das aves aos 7 dias de idade (Dal Pont *et al.*, 2019). Sabe-se que o desempenho das aves da primeira semana de vida está altamente correlacionado ao desempenho final do ciclo produtivo (Willemsen *et al.*, 2008). Assim, acredita-se que os efeitos benéficos do glicerol poderiam ser prolongados até a idade de abate. Atualmente, não há relatos na literatura avaliando os efeitos da injeção de glicerol sobre esses parâmetros.

Alguns dos principais problemas que podem prejudicar o desempenho inicial da ave é a limitação do trato gastrointestinal. Assim, é desejável que o trato gastrointestinal se torne fisiologicamente maduro o mais cedo possível (Uni *et al.*, 2003). Melhorias das condições do epitélio intestinal dos pintos após a eclosão foram observadas após a inoculação *in ovo* de glicerol (Neves *et al.*, 2016). Até o presente momento, há poucos relatos na literatura que elucidam os efeitos da injeção de glicerol *in ovo* sobre o epitélio intestinal, o que torna importante a avaliação desse nutriente na nutrição *in* ovo.

Outra característica benéfica do glicerol está associada à reabsorção de água pelos rins e à capacidade de reter líquido pelo organismo (Montner *et al.*, 1999). Como na prática as aves permanecem sem acesso à alimentação e à água, algumas vezes, por até 72 horas, acredita-se que a injeção *in ovo* de glicerol possa minimizar a desidratação dos pintos recém-eclodidos.

Em suma, como o glicerol é o principal precursor para a gliconeogênese, em especial no final do desenvolvimento embrionário, com potencial de ser utilizado pelos embriões para gerar glicose e consequentemente energia, supõe-se que a inoculação *in ovo* de glicerol favoreça o embrião antes da eclosão, além de promover melhorias no desempenho das aves até o final do ciclo produtivo. Contudo, são necessárias mais pesquisas para esclarecer os seus efeitos, bem como avaliar a melhor dose a ser aplicada na nutrição *in ovo*.

2.4. O potencial do IGF-I como substância a ser utilizada na nutrição *in ovo*

Os fatores de crescimento semelhantes à insulina (IGF) compreendem uma família de hormônios polipeptídicos estruturalmente semelhantes à insulina (Mcmurtry *et al.*, 1997). O sistema do IGF é composto pelo IGF-I e IGF-II, receptores de IGF do tipo I e do tipo II, proteínas de ligação ao IGF (IGFBPs) e proteases de IGFBP (Allan *et al.*, 2001). A composição de aminoácidos do IGF-I em frangos é semelhante à de patos (Kansaku *et al.*, 2003) e perus (Czerwinski *et al.*, 1998) e diferem em oito aminoácidos dos humanos (Rinderknecht e Humbel, 1978), bovinos (Francis *et al.*, 1988) e suínos (Francis *et al.*, 1989). O receptor do IGF-I do frango possui 85% de semelhança ao receptor humano (Holzenberger *et al.*, 1996). Embora possa haver diferenças na composição de aminoácidos dos IGFs e de seus receptores entre mamíferos e aves, os efeitos provocados na sua presença são homólogos. Os IGFs são expressos em todos os tecidos e desempenham papéis cruciais na proliferação, diferenciação e metabolismo celular (Stewart e Rotwein, 1996). Além dos efeitos endócrinos exercidos pelos IGF's circulantes na corrente sanguínea, os IGFs produzidos localmente atuam de forma parácrina e autócrina sobre a proliferação celular (Jones e Clemmons, 1995).

Durante o desenvolvimento embrionário, o IGF-I começa a ser expresso ainda no estágio de blastoderme, ovo recém-colocado (Serrano *et al.*, 1990). As baixas concentrações do hormônio de crescimento sugerem que o mesmo não seja responsável pelo estímulo do crescimento embrionário (Hazelwood, 2000). A independência do hormônio de crescimento e a expressão precoce do IGF-I indicam que esse peptídeo desempenha papel significativo no crescimento de embriões (Kocamis *et al.*, 1998).

O nível plasmático de IGF-I aumenta do dia 6 para um pico no 15° dia de incubação e diminui até a eclosão (Lu *et al.*, 2007). Até o 15° dia de incubação, o embrião não possui o sistema circulatório maduro e a ação do IGF-I é realizada de forma parácrina e autócrina (Allan *et al.*, 2001). O pico está relacionado à maturação dos tecidos e a capacidade dos mesmos de sintetizar o IGF-I. Já o declínio após o 15° dia está relacionado à mudança do metabolismo energético, quando o glicogênio passa a ser a fonte primária de energia (Mcmurtry *et al.*, 1997). De fato, estudos mostram que o IGF-I atua como a insulina e pode estimular a síntese de glicogênio hepático (Parkes *et al.*, 1986; Mcmurtry, 1998; Lu *et al.*, 2007).

Outra característica importante é que o número de fibras musculares não aumenta após a eclosão e é determinado apenas durante o período embrionário (Liu *et al.*, 2011). A

hipertrofia muscular após a eclosão é devido ao alongamento e espessamento das fibras musculares (Van Der Ven *et al.*, 1991). Portanto, os fatores que influenciam o desenvolvimento das fibras embrionárias podem afetar o crescimento muscular após a eclosão.

Nesse contexto, o IGF-1 desempenha papel fundamental no estímulo da proliferação e da diferenciação de células musculares durante a miogênese (Sandri *et al.*, 2013). Tem sido demonstrado que a ação mitogênica do IGF-1 nas células musculares é essencial e mediada pela via da fosfatidilinositol 3-quinase (PI3K)/proteína quinase B (Akt) (Sandri *et al.*, 2013; Yu *et al.*, 2015). A ativação dessa via está relacionada à progressão do ciclo celular e à sobrevivência das células (Liu *et al.*, 2011). Logo, o IGF-1 requer a via de sinalização PI3K/Akt para a proliferação de mioblastos e para aumentar o número de fibras musculares (Yu *et al.*, 2015). Estudos relatam que as concentrações plasmáticas de IGF-I estão positivamente correlacionadas com as taxas de crescimento muscular em aves (Tomas *et al.*, 1998; Lu *et al.*, 2007). Assim, a maximização no desenvolvimento e crescimento muscular poderia ser obtida como aumento na disponibilidade desse peptídeo por meio da injeção *in ovo*.

A inoculação de 500 ng/embrião de IGF-I nos dias 7 e 14 do desenvolvimento embrionário não estimulou o crescimento e nem aumentou o peso individual de órgãos ou de tecidos das aves no momento da eclosão (Spencer *et al.*, 1990). Ao contrário, foi demonstrado que, quando administrado precocemente no dia 2, o IGF-I (100 ng/embrião) estimula vários indicadores metabólicos e de crescimento, quando avaliado 48 horas depois (Girbau *et al.*, 1988). Entretanto, os embriões não foram incubados até a eclosão para verificar se esses efeitos benéficos foram prolongados. Por outro lado, Liu *et al.* (2011), estudando o desenvolvimento embrionário de patos provenientes de ovos inoculados com 100 ng/embrião de IGF-I aos 12 dias de incubação, observaram maior peso dos embriões aos 27 dias de incubação e nas aves 2 dias após a eclosão. Maior desenvolvimento do músculo esquelético também foi observado em codornas com a inoculação de 100 ng/embrião de IGF-I no terceiro dia de incubação (Deprem e Gülmez, 2007). Kocamis *et al.* (1998) observaram maior ganho de peso e melhor conversão alimentar em frangos de corte aos 42 dias de idade, quando foram inoculados com 100 ng/embrião de IGF-I nos ovos no terceiro dia de incubação. Mohammadrezaei *et al.* (2014) também relataram maior ganho de peso e menor conversão alimentar aos 42 dias em frangos de corte após terem sido inoculados com 100 ou 200 ng/embrião de IGF-I no quinto dia de incubação dos ovos.

O IGF-I também tem sido relacionado à estimulação do crescimento da mucosa intestinal e a maturação de suas células (Jehle *et al.*, 1999). A expressão do RNAm de IGF-I no intestino de frangos, patos e perus antes e após a eclosão foi observada (Karcher *et al.*, 2009). Em patos, aumento na diferenciação morfológica após a eclosão do intestino delgado foi relatado com a inoculação de fatores de crescimento realizada no 13º dia de incubação no líquido amniótico (Wang *et al.*, 2012). Melhora da atividade enzimática no intestino foi obtida em frangos de corte com 21 e 42 dias de idade provenientes de ovos injetados com 100 ng/embrião IGF1 no quinto dia de incubação (Moosavinasab *et al.*, 2015).

Em suma, por estar relacionado a proliferação, diferenciação e metabolismo celular, supõe-se que a inoculação *in ovo* de IGF-I poderia aumentar a síntese de glicogênio e favorecer o embrião antes da eclosão, promover melhorias no epitélio intestinal, aumentar o número de fibras musculares e, consequentemente, melhorar as características de carcaça das aves. Contudo, há poucos estudos avaliando os efeitos do IGF-I sobre o desenvolvimento de frangos de corte, assim, são necessárias mais pesquisas para esclarecer esses efeitos, bem como, identificar o melhor momento e a melhor dose a ser aplicada na nutrição *in ovo*.

3. CONSIDERAÇÕES FINAIS

O contínuo desenvolvimento e aprimoramento da tecnologia *in ovo* estabeleceu um novo escopo para a nutrição perieclosão. Este fato permite e cria novos desafios e oportunidades para que os nutricionistas otimizem a produção de aves. A injeção *in ovo* de nutrientes ou substâncias no âmnio é uma nova maneira de melhorar o estado nutricional do embrião impulsionando o seu desenvolvimento. Como mencionado ao longo da revisão, a técnica de alimentação *in* ovo apresenta várias vantagens, porém, as principais limitações ainda estão associadas ao desenvolvimento embrionário e ao metabolismo de nutrientes. Mais pesquisas são necessárias para compreender como a inoculação de nutrientes influencia o desenvolvimento embrionário, o metabolismo desses nutrientes e entender como a nutrição precoce afeta os genes responsáveis pelo desenvolvimento das aves.

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SEGUNDA PARTE - ARTIGOS

ARTIGO 1 - *In ovo* feeding of glycerol on the development of broiler chickens

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Short title: *In ovo* feeding of glycerol for broilers

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Abstract

This study was conducted to evaluate the effects of *in ovo* feeding of glycerol on hatchability, biochemical parameters, intestinal morphometry, performance and carcass characteristics of broilers. A total of 360 broiler fertile eggs were distributed to six experimental groups consisting of one control (non-injected) and five saline solutions (0.9%) injected into the amnion at the 17th day of incubation containing 0, 10, 20, 40 or 80 nmol/mL of glycerol. The results showed that the hatchability was not affected (P>0.05) among groups. The doses of 10, 20 or 40 nmol/mL of glycerol reduced (P<0.01) the incubation time. Eggs that were injected with 20, 40 or 80 nmol/mL of glycerol hatched chicks with lower glycemia (P<0.01) in comparison to those of control groups and no effect (*P*>0.05) of glycerol injection was observed on the plasma uric acid. The dose of 80 nmol/mL of glycerol decreased (P=0.01) the muscular glycogen and did not influence (P>0.05) the hepatic glycogen. There was no effect (P>0.05) on hepatic glycerol kinase activity and higher (P=0.02) fructose 1,6-bisphosphatase activity was observed at doses of 40 or 80 nmol/mL of glycerol. Intestinal morphometry was not influenced (P>0.05) by the injection of solutions containing different concentrations of glycerol. The use of 20 or 40 nmol/mL of glycerol increased (P=0.01) the relative weight of the duodenum at 1 and 7 days of age. The growing concentrations of glycerol increased (P<0.05) the relative breast weight at 7 days of age and the deposition of abdominal fat at 40 days of age. At 7, 14 and 21 days of age, the eggs that were injected with 10 or 20 nmol/mL of glycerol that resulted broilers with better performance (P<0.05). It is concluded that 10 or 20 nmol/mL of glycerol can be used as a substrate in *in ovo* feeding of broilers to improve performance until the third week, without influencing hatchability.

Keywords: poultry, *in ovo* nutrition, energy substrate, embryo development, metabolism

Implications

The *in ovo* feeding technique established a new strategy in early nutrition and has been indicated to improve the production efficiency of broilers. In the present study, we observed that *in ovo* feeding with glycerol improved some metabolic aspects of hatching broilers, which reflected in improved broiler performance during the first three weeks. Although no effects on the performance and carcass characteristics were observed at the end of the production cycle, these findings represent a basis for future studies in relation to the use of other concentrations and the ideal time for the technique to be performed.

Introduction

The hatching weight and the initial performance of the chicks have a great influence on the body weight and yield of cuts at slaughter (Chen *et al.*, 2013, Al-Nedawi *et al.*, 2018, Yu *et al.*, 2018). During the embryonic phase, the embryo makes use of the nutrients contained in the egg (Yadgary and Uni, 2012). However, due to the high genetic potential of birds, it is believed that the nutrients contained in the egg, especially carbohydrates, may be insufficient and limit the development of birds before and post-hatch (Retes *et al.*, 2018). In addition, the energy deficit in the period before hatching can increase embryonic mortality and decrease numbers of hatching eggs (Moreira Filho *et al.*, 2018).

The glucose is the main energy substrate of the animal cell (Shafey *et al.*, 2012). As the amount of this substance is limited in the egg, maintenance of

glycemia is dependent on the amount of glucose contained in glycogen stores and hepatic gluconeogenesis. At the end of incubation, the glycogen stores are depleted (Zhao *et al.*, 2017) and the embryo uses amino acids as the gluconeogenic substrate, reducing the availability of these nutrients for muscle synthesis (Chen *et al.*, 2009, Noy and Uni, 2010). As a consequence, development post-hatch can be compromised (Uni and Ferket, 2004). In addition, it is known that the glycogen stores of the embryo are also related to the body weight (Uni *et al.*, 2005) and the development of the immune system (Dibner *et al.*, 1998).

In an attempt to improve the post-hatch development, the supply of nutrients during the incubation has been tested (Uni *et al.*, 2005, Yu *et al.*, 2018, Zhao *et al.*, 2018). However, even though various substrates may serve as precursors for glucose and glycogen synthesis, their preference varies with the availability, the embryonic stage of development, and the enzyme localization (Sunny, 2008). Studies that evaluated the effects of *in ovo* injection of glucose (Ipek *et al.*, 2004, Leitão *et al.*, 2008, Zhang *et al.*, 2016) observed reduced hatchability and lack of performance improvement post-hatch.

It is well documented that glycerol is an important precursor for gluconeogenesis (Sunny and Bequette, 2011). It is believed that *in ovo* feeding of this substance should reduce the use of amino acids to maintain glycemia and favor hepatic and muscle glycogen stores. Recent studies have shown that injection *in ovo* of glycerol between 17 th and 18 th day of incubation improved intestinal epithelial the conditions and increased the hepatic glycerol kinase activity (Neves *et al.*, 2016) and the hepatic glycogen store of newly hatching chicks (Dal Pont *et al.*, 2019).

The evaluated use of glycerol in *in ovo* feeding on performance and carcass characteristics at different ages of broiler chickens post-hatch has not been reported

in the literature. The hypothesis is that the inoculation of this substance can improve the metabolic parameters and the absorption efficiency in the small intestine, resulting in more productive broilers. Thus, the objective of this study was to evaluate the effect of *in ovo* feeding with glycerol on the biochemical parameters, hatchability, intestinal morphometry, performance and carcass characteristics of broilers.

Material and Methods

Incubation

This experiment was approved by the Institutional Animal Care and Use Committee of Federal University of Lavras (Lavras, Brazil), under protocol number 49/14. A total de 480 eggs, from the Cobb 500® broiler breeder flocks of 55 weeks, were obtained from the commercial hatchery. All eggs were selected and weighed individually with an average weight of 70.9± 3.6 g. Then, the eggs were pre-heated to 30 °C for 12 hours and were randomly distributed in the six incubator tray levels (Luna 480, Chocmaster, Piraquara, Brazil) and incubated at temperature of 37.5 ± 0.1 °C with relative humidity of 60 ± 0.9%. All trays contained all the experimental groups. At 17 days of incubation, eggs were candled for selecting embryonated eggs and removing the ones unfertilized or non-viable. After examination, 360 embryonated eggs were distributed to six experimental groups of 60 fertile eggs in a completely randomized design.

Inoculated solutions

The experimental groups consisted of a control group (non-injected) and five 0.9% saline solutions injected groups with different concentrations of glycerol (≥99% glycerol, Sigma Aldrich, Darmstadt, Germany): 0, 10, 20, 40 and 80 nmol/mL, totaling

six experimental groups. The solutions to be injected contained pH 7.30 ± 0.2 (pH meter W3B; Bel Engineering, Monza, Italy) and osmolarity 312.5 ± 14.0mOsm (osmometer K-7400, Kanauer, Berlin, Germany).

On 17th day of incubation, all injected solutions were freshly prepared and kept in the incubator at 30 °C to avoid thermal shock to the embryo. The location of the amnion was identified by candling, the injection place was disinfected with with 2% iodinated alcohol and 0.5 ml solution was injected into the amnion using 22-gauge needle. After each injection, the needles were replaced. The control group received no injection, but it was subjected to the same handing procedures as the injected groups. Immediately after the injection, the holes were sealed with paraffin wax (Gonzales et al., 2013) and eggs were placed in bride sacs containing the identification of the experimental group (Pedroso et al., 2006). Then, the eggs were returned to the incubator and incubated with conventional procedure to complete the hatching process. The whole process was carried out in a sanitized room that was maintained at an average temperature of 30 °C. All eggs were held outside the incubator for less than 1 hour to complete the injection process, including the non-injected control group. It is noteworthy that to ensure that the injection site was the amniotic fluid, 15 embryonated test-eggs were injected with a water-soluble dye and then broken to verify the injected site.

Animal husbandry

The hatching times were registered. At each examination period, the numbers of chicks hatched were counted and the hour registered. After 26 days of incubation, the non-hatched eggs were opened and the embryo mortality period was classified before and after inoculation by embryodiagnosis (Cobb, 2013). Hatchability was

obtained by dividing the number of eggs that hatched with viable chicks by the number of fertile eggs on the 17th day of incubation.

Upon hatch, the chicks were housed separately, according to the experimental group, in 2.0 m long × 1.5 m wide boxes with floors covered with shavings, a tubular feeder and an infant-type drinker, which was replaced 7 days later by the pendulum type; the boxes were in a screened masonry shed pre-heated with infrared lamps. Chicks were allowed free access to feed and water. When there were no more hatched eggs (48 hours after the first bird had hatched), each chick birds was weighed, sexed and housed in mixed lots. The experimental design was a completely randomized block (initial weight and male:female ratio), with six treatments with five replicates of 5 ± 1 birds each.

Continuous fluorescent illumination was maintained, and the temperature of the experimental room was monitored through two thermohygrometers (Simpla TH02, Asko, São Leopoldo, Brazil) placed in the shed at the height of the birds. The ingredients and nutrient levels of diets were formulated to meet the nutrient requirements (Rostagno et al., 2011) (Table 1). The chicks were raised until 40 d of age. At 7, 14, 21, 35 and 40 days, birds and feed were weighed, and feed intake was recorded by replicate to calculate the weight gain, feed intake and feed/gain ratio.

Sample collection

Upon hatch, randomly selected eight male birds per experimental group were individually weighed and euthanized by cervical dislocation. Blood samples were collected during bleeding. After collection, the blood was centrifuged at 1000 × g for 15 minutes (Sorvall TM ST16 Centrifuge, Thermo Fisher Scientific, Massachusetts,

USA) to obtain plasma. The plasma was stored at -80 °C for glucose and uric acid analysis.

From the same sample chicks, the weights of heart, liver, gizzard, yolk sac, breasts, thigh plus drumstick and small and large intestine were determined. The liver and breast were immersed in liquid nitrogen for a few seconds and stored at -80 °C for fructose-1,6-bisphosphato phosphatase (FBP) and glycerol kinase activity and hepatic and muscular glycogen determination. The segments from the duodenum, jejunum and ileum were flushed with physiological saline and fixed in Bouin solution for 24 h. At 7 d of age, two male birds from each box were selected, according to the average weight of the box. These birds were euthanized following the same procedure described for hatch chicks.

At 40 d of age, two male birds with similar weights to the box average were selected and slaughtered, feathered and eviscerated. The relative weight of carcass (without legs and abdominal fat), viscera, breast (skin and bone), wings and leg (thigh plus drumstick with skin and bone) were calculated in relation to live weight.

Biochemical analyses

Glucose and uric acid were quantified by an enzymatic colorimetric test (Liquiform Glucose, Liquiform Uric Acid, Labtest, Lagoa Santa, Brazil), following the manufacturer's recommendations.

The activity of liver glycerol kinase was assessed using an adaptation of the methodology presented by Sakasegawa et al. (1998). For this, ± 0.2 g of frozen liver tissue was homogenized in 1 mL of buffer solution (50 mM Tris-HCl, pH 8.0). After tissue homogenization, the sample was centrifuged (22500x g for 20 minutes at 5 °C)

and the supernatant was collected. Glycerol kinase activity was expressed as moles of glycerol-3-phosphate formed/min/mg protein.

For FBP activity, the liver (±0.2 g) was homogenized in 1 mL of solution containing 0.1 M Tris-HCl (pH 7.5), 0.15 M KCl, 5 mM dithiothreitol and 5 mM $MgSO_4$. After tissue homogenization, the sample was centrifuged (20600 × g for 60 minutes at 4 °C) and the supernatant was collected according to the methodology proposed by Aoki et al. (1999). The determination of the enzymatic activity was performed according to Ulm et al. (1975).

The hepatic and muscular glycogen contents were determined according to Willems et al. (2014). For this, liver and breast fragments (± 0.2 g) were homogenized in 7% perchloric acid solution. After complete homogenization of the tissue, the sample was centrifuged (14000 × g for 15 minutes at 4 °C) and the supernatant was collected. Quantification was determined by color reagent based on iodine.

Intestinal morphometry

Samples of the duodenum, jejunum and ileum with approximately 5 mm in length were subjected to routine histological procedures, being dehydrated in solutions with increasing concentrations of ethanol, cleared in xylene, embedded in paraffin, microtomized to 4.0 µm, arranged on silanized glass slides, dried at 37 °C overnight and stained with haematoxylin and eosin (Suvarna et al., 2018). Two cuts of different regions were performed for each intestinal segment, of which scanned images were analyzed using an Olympus microscope (CX31; Olympus, Tokyo, Japan) coupled to an Altra SC30 digital camera (Olympus) using the AxioVision program (Carl Zeiss, Oberkochen, Germany) at 100× magnification for the

duodenum, 200× for the jejunum and ileum to birds at one day of age and 100× for the different segments to birds seven days of age. Ten readings were taken per slide, measuring villus height and crypt depth. Villus height was measured from the tip of the villi to the villus crypt junction and crypt depth was defined as the depth of the invagination between adjacent villi. The average value was calculated per variable per chick. Later, villus: crypt ratio was calculated (VH/CD).

Statistical analysis

The hatchability data were submitted to a binomial model and the main effect (treatments) was analyzed by a likelihood ratio test. Means were compared by a Pearson Chi-square test (Leitão et al., 2010). The other data were submitted to tests for normality (Shapiro–Wilk), homoscedasticity of variance (Breusch–Pagan) and independence of errors (Durbin–Watson). In the case of non-significance on these tests, an analysis of variance (ANOVA) was performed and glycerol levels were submitted to a regression analysis. The means obtained with each level of glycerol were compared to the control (intact eggs) by a Dunnett's test. When there was no adjustment of the regression curve (R_2<0.70), the means were compared by a Scott–Knott test at 5%. For the variables that did not meet the ANOVA assumptions, the Box-Cox or Johnson data transformation options were used. The variables that did not reach normality, even after data transformation, were submitted to non-parametric analysis and the means were compared by a Kruskal–Walis test. All statistical analyzes were performed in the statistical program Action version 3.4 (Estatcamp, São Carlos-SP, Brazil).

Results

Hatchability and Biochemical Parameters

There was no effect (*P>0.05*) on hatchability among *in ovo* feeding groups (Figure 1, B). The injection of solutions with 10, 20 or 40 nmol/mL of glycerol decreased (*P <0.01*) the incubation time (Figure 1, A).

The injection of solutions with 20, 40 or 80nmol/mL glycerol reduced (*P<0.05*) the glycemia in comparison to those of control groups (Figure 2, A). There was no effect (*P> 0.05*) on the concentration of plasma uric acid (Figure 2, B). The injection with 80 nmol/mL glycerol decreased (*P=0.01*) muscle glycogen in comparison to those of non-injected groups (Figure 2, C), but did not influence (*P>0.05*) the hepatic glycogen (Figure 2, D). Higher (*P=0.02*) fructose 1,6-bisphosphatase activity was observed with doses of 40 or 80 nmol/mL glycerol (Figure 2, E). The hepatic glycerol kinase activity was not influenced (*P> 0.05*) by *in ovo* injection of the solutions (Figure 2, F).

Intestinal Morphometry

Intestinal morphometry was not influenced (*P>0.05*) by the injection of solutions containing different concentrations of glycerol (Table 2).

Performance

At 7 days, higher feed intake and weight gain were observed (*P<0.01)* with injection of 10 or 20 nmol/mL glycerol in comparison to those of control groups (Table 3). Worse feed conversion was observed (*P=0.02*) when the injection with saline solution or with 40 nmol/mL glycerol was used.

At 14 days, higher weight gain was observed (*P<0.01*) with injection of 10 or 20 or 40 nmol/mL glycerol in comparison to those of control groups. At 21 days, higher weight gain was observed (*P=0.03*) with injection of 10, 20 or 80 nmol/mL glycerol. There was no effect (*P>0.05*) on broiler performance at 39 days of age.

Body composition

The injection of solutions with 20 or 40 nmol/mL of glycerol increased (*P<0.05*) the relative weight of the duodenum at 1 and 7 days of age (Table 4). Higher relative breast weight was observed (*P=0.02*) at 7 days of age in chicks from eggs injected with solutions containing the different concentrations of glycerol and non-injected. At 40 days of age, injection of solutions containing glycerol increased (*P=0.02*) the deposition of abdominal fat. There was no effect (*P>0.05*) of injection with glycerol on the other carcass characteristics evaluated.

Discussion

Injection of nutritive solutions *in ovo* has been associated with an improvement of the performance of broiler after hatch. In the present study, the injection of solutions containing 10 or 20 nmol/mL glycerol improved the weight gain in the first three weeks of life, without influencing hatchability.

It is known that the first seven days of age are considered critical for the development of birds (Almeida *et al.*, 2006) since the performance in this phase is highly correlated to the final performance of the productive cycle (Willemsen *et al.*, 2008). Although in the present study no improvement was observed in performance at 39 days of age, an increase of approximately 15% was observed in the weight gain of broilers at 21 days with the injection of 10 or 20 nmol/mL glycerol. This result

may be related to changes in metabolism in the final period of embryonic development (Moreira Filho *et al.*, 2018). According to Sunny and Bequette (2011), glycerol is the main precursor of gluconeogenesis and of glycogen and nonessential amino acids synthesis. Thus, greater weight gain in the first three weeks of life may be associated with the use of glycerol by the embryos as a gluconeogenic substrate in the final stage of embryonic development, reducing the use of amino acids and increasing the contribution of these nutrients to protein synthesis (McMurtry, 1998, Lu *et al.*, 2007). This theory could also explain the higher relative breast weight observed at 7 days of age in chicks from eggs injected with solutions containing the different concentrations of glycerol.

The increase of fructose 1,6-bisphosphatase activity in newly-hatched chicks from eggs injected with 40 or 80 nmol/mL glycerol suggests increased gluconeogenic activity at the end of the hatching period (Chaekal *et al.*, 1983). According to Prado-Rebolledo *et al.* (2009), the high muscular activity required for bark pecking combined with the low O_2 tension present until the onset of respiration by the air sacs requires a higher supply of glucose to obtain energy. During this phase, gluconeogenesis depends on glycerol, lactate and amino acids (Watford *et al.*, 1981), reducing the availability of these latter's for muscle synthesis (Chen *et al.*, 2009, Noy and Uni, 2010). However, the results of the plasma uric acid obtained in the present study indicate that there were no differences in amino acid catabolism. Thus, increased fructose 1,6-bisphosphatase activity and lack of effects on plasma uric acid suggest that the supposed increase in the metabolic activity provided by doses greater than 40 nmol/mL glycerol stimulated gluconeogenesis but not from amino acids. As the glycerol kinase activity did not increase with the use of solutions containing higher concentrations of glycerol, it is believed that lactate may have been

the main gluconeogenic precursor of these embryos in the final stage of embryonic development (Watford *et al.*, 1981). In this period, the increase in the muscle metabolic rate due to the bark pecking process associated to the increase in the respiration rate, results in the decrease of O_2 and, consequently, a greater formation of lactate by the anaerobic metabolism. In addition, the reduction of O_2 tension is associated with the increase in CO_2 concentrations within the egg (Khaligh *et al.*, 2017) and the elevation of that gas may anticipate the hatching (Everaert *et al.*, 2007).

The results of the present study, with the exception of the higher dose, showed that glycerol reduced the incubation time and narrowed the hatch window, the interval between the first and last egg to hatch (Araújo *et al.*, 2016). However, the results obtained differ in relation to the amount of plasma glucose. Higher levels were obtained with the lower dose of glycerol, while lower levels were observed with the injection of doses higher than 20 nmol/mL glycerol. It is believed that in the attempt to supply energy demand for the hatching process, the embryos supplemented with the highest dose of glycerol used muscle glycogen for hatching process. As the hatching was not affected, it is believed that the amount of glucose in the blood was necessary to meet the energy demand of the hatching process.

Still, on the hatch window, it is known that birds do not hatch at the same time, which makes it difficult to determine the ideal time for chicks to be removed (Decuypere *et al.*, 2001). If removal of the chicks from the hatcher is carried out in advance, fewer hatched eggs can be obtained. On the other hand, if the chicks are removed late, early hatched chicks may suffer dehydration and decrease their energy reserves, impairing the performance of broilers (Careghi *et al.*, 2005). Thus, a narrower hatch window is desirable and result in quality chicks (Careghi *et al.*, 2005,

Joseph and Moran, 2005). In this case, the *in ovo* feeding with glycerol could be used to promote the reduction of the hatch window and, consequently, produce quality day-old chicks, as well as provide improvements in the dynamics of the hatchery.

In the hatchery, because they are restricted to water and feed, the chicks lose around 4 g of body weight every 24 hours, due to the loss of moisture and the use of nutrients contained in the yolk (Noy and Sklan, 1998, Batal and Parsons, 2002). This delay in access to food and water are some of the main problems that can compromise the performance, slaughter weight and cuts yield. Early hatched chicks were not able to compensate for body weight loss after hatching and had performance compromised at 35 days of age (El Sabry *et al.*, 2013). On the other hand, improvements in the performance of broiler at 35 days of age from eggs injected with solution containing dextrin and beta-hydroxy-beta-methylbutyric (HMB) were superior to the performance of the birds subjected to the feed restriction for 36 hours (Kornasio *et al.*, 2011). Therefore, because the broilers in the present study were not subjected to food and water restriction, it is believed that the beneficial results observed in the first three weeks could be prolonged until the final of the production cycle and, consequently, promote improvements in slaughter weight and cut yields.

At the final of the production cycle, the increase in abdominal fat observed suggests the influence of glycerol on the lipid metabolism of birds. As the low activity of glycerol kinase in adipose tissue, the generation of glycerol 3-phosphate by glycerol-3-phosphate dehydrogenase is the primary source of triglyceride synthesis (Swierczynski *et al.*, 2003, Sledzinski *et al.*, 2013). The growth rate of adipose tissue deposits increases according to the activity of glycerol-3-phosphate dehydrogenase (Bai *et al.*, 2015). Thus, it is believed that not used glycerol in the energetic demand

of the hatching process promoted an increase in glycerol-3-phosphate dehydrogenase activity and increased abdominal fat.

In ovo feeding has been shown to be a viable technique to be applied in commercial hatcheries. The positive results observed in the present study may be related to the fact that glycerol was used by the embryos and had decreased the use of amino acids as a gluconeogenic substrate before hatching, thus increasing the availability of these last nutrients for muscle growth. It is concluded that 10 or 20 nmol/mL glycerol can be used as a substrate in *in ovo* feeding of broilers to improve performance until the third week, without influencing hatchability.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

Ethics statement

The protocol applied to animal experiments was approved (protocol number 49/14) by the Institutional Animal Care and Use Committee of Federal University of Lavras (Lavras, Minas Gerais, Brazil).

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547 in ovo feeding of creatine pyruvate on the hatchability, growth performance and
548 energy status in embryos and broiler chickens. Animal 11, 1689-1697.

549 **Table 1** Centesimal composition and calculated nutritional levels of the experimental
550 diets used during the different stages of the post-hatch development of broilers.

Ingredient	1-7 days	8-21 days	22-35 days	36- 40 days
Corn	55.489	59.645	62.377	67.093
Soybean meal (45%)	38.162	34.664	31.437	27.218
Soy oil	2.055	2.013	2.963	2.787
Dicalcium phosphate	1.907	1.508	1.273	1.070
Calcitic limestone	0.914	0.924	0.865	0.771
Salt	0.507	0.482	0.457	0.444
DL-Methionine (99%)	0.359	0.287	0.256	0.240
L-Lysine (78%)	0.289	0.219	0.196	0.237
L-Threonine (99%)	0.114	0.064	0.043	0.053
Salinomycin	0.050	0.050	0.050	0.000
Vitamin supplement [1]	0.050	0.040	0.030	0.020
Mineral supplement [2]	0.050	0.050	0.050	0.050
Choline chloride (60%)	0.050	0.050	0.000	0.020
Avilamycin	0.005	0.005	0.005	0.000
TOTAL	100.00	100.00	100.00	100.00
Calculated nutritional composition				
Metabolizable energy, kcal/kg	2950	3000	3100	3150
Crude protein, %	22.20	20.80	19.50	18.00
Calcium, %	0.920	0.819	0.732	0.638
Available phosphorus, %	0.470	0.391	0.342	0.298
Digestible lysine, %	1.310	1.174	1.078	1.010
Digestible methionine + cystine, %	0.944	0.846	0.787	0.737
Digestible threonine, %	0.852	0.763	0.701	0.656
Sodium, %	0.220	0.210	0.200	0.195

551 1. Supply per kg of product: 5.000.000 IUof vitamin A; 1.850.000IUof vitamin D3; 4.500 IUof vitamin E;
552 918 mg of vitamin K3; 2.000 mg of vitamin B2; 250 mg of vitamin B6; 6,500 mcg of vitamin B12; 145.4
553 mg of folic acid; 12.9 g of niacin; 5,931 mg of pantothenic acid and 480 mg of selenium.
554 2. Supply per kg of product: 67.5 g of manganese; 50.4 g of iron; 43.2 g of zinc; 7.0 g of cupper and
555 1,464 mg of iodine.

Table 2 Intestinal epithelium morphometry of different aged broilers from non-injected eggs and injected eggs with solutions containing different concentrations of glycerol.

Variable	Non-Injected	Saline solution	Glycerol (nmol/mL)				SEM	P value
			10	20	40	80		
1 day of age								
Duodenum								
Villus height (µm)	356	382	403	408	407	390	6.98	0.42
Crypt depth (µm)	79	75	64	70	74	76	1.57	0.27
Villus:Crypt	4.7	5.2	6.5	6.0	5.5	5.3	0.14	0.07
Jejunum								
Villus height (µm)	207	195	230	221	225	244	4.82	0.26
Crypt depth (µm)	54	64	55	62	60	60	1.30	0.43
Villus:Crypt	3.8	3.7	4.2	3.7	3.8	4.1	0.10	0.13
Ileum								
Villus height (µm)	172	181	187	216	214	188	3.90	0.07
Crypt depth (µm)	52	64	72	58	56	52	2.11	0.15
Villus:Crypt	3.6	3.1	2.8	3.9	3.9	3.9	0.13	0.18
7 days of age								
Duodenum								
Villus height (µm)	550	600	682	562	641	623	16.99	0.48
Crypt depth (µm)	143	122	123	124	135	120	3.45	0.64
Villus:Crypt	4.1	4.9	5.6	4.4	4.9	5.2	0.12	0.09
Jejunum								
Villus height (µm)	533	469	522	548	529	597	15.95	0.74
Crypt depth (µm)	113	95	124	116	131	127	3.22	0.21
Villus:Crypt	4.9	5.0	4.3	4.9	4.1	4.8	0.12	0.31
Ileum								
Villus height (µm)	424	400	447	462	456	404	9.25	0.51
Crypt depth (µm)	98	88	96	99	103	89	3.62	0.93
Villus:Crypt	4.4	4.9	4.9	4.9	4.6	4.9	0.14	0.91

SEM: standard error of the mean

560 **Table 3** Performance of broilers from non-injected eggs and injected eggs with
561 solutions containing different concentrations of glycerol.

Performance	Non-injected	Saline solution	Glycerol (nmol/mL)				SEM	P value
			10	20	40	80		
1 to 7 days of age								
Feed intake (g)	82 b	81 b	100 a	107 a	101 a	74 b	1.949	<0.01
Weight gain (g)	86 b	71 c	101 a	109 a	88 b	77 c	1.974	<0.01
Feed conversion	0.96 b	1.13 a	0.99 b	0.99 b	1.15 a	0.98 b	0.021	0.02
1 to 14 days of age								
Feed intake (g)	461.3	463.6	500.9	523.9	502.5	490.1	7.91	0.13
Weight gain (g)	373 b	394 b	427 a	449 a	424 a	405 b	5.43	<0.01
Feed conversion	1.24	1.18	1.17	1.17	1.18	1.22	0.02	0.74
1 to 21 days of age								
Feed intake (g)	1042	1069	1115	1200	1109	1156	14.72	0.13
Weight gain (g)	781 b	846 b	903 a	916 a	823 b	869 a	12.96	0.03
Feed conversion	1.33	1.27	1.24	1.31	1.35	1.33	0.02	0.18
1 to 39 days of age								
Feed intake (g)	3124	3397	3368	3340	3292	3411	64.96	0.75
Weight gain (g)	2153	2360	2456	2447	2249	2389	45.03	0.26
Feed conversion	1.48	1.44	1.38	1.37	1.47	1.43	0.03	0.82

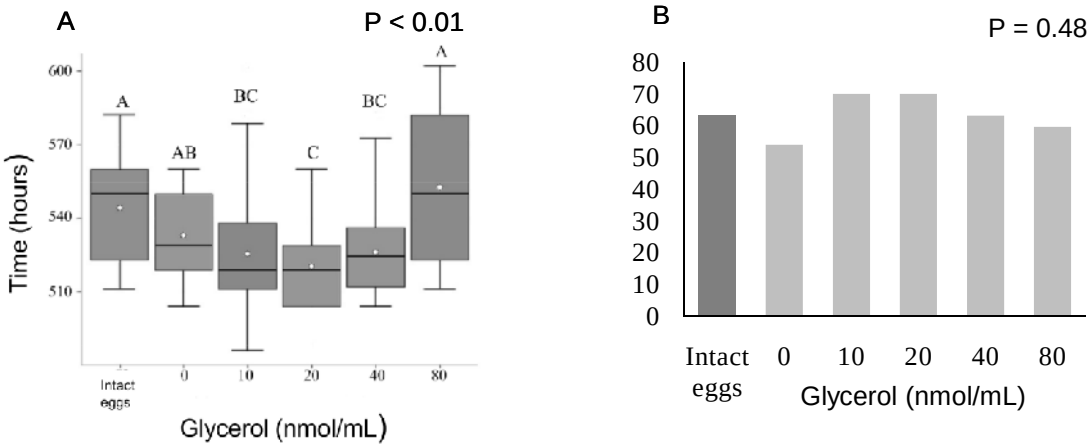
562 SEM: standard error of the mean

563 [a,b] Means followed by different letters within a row differ by a Scott–Knott test (P<0.05)

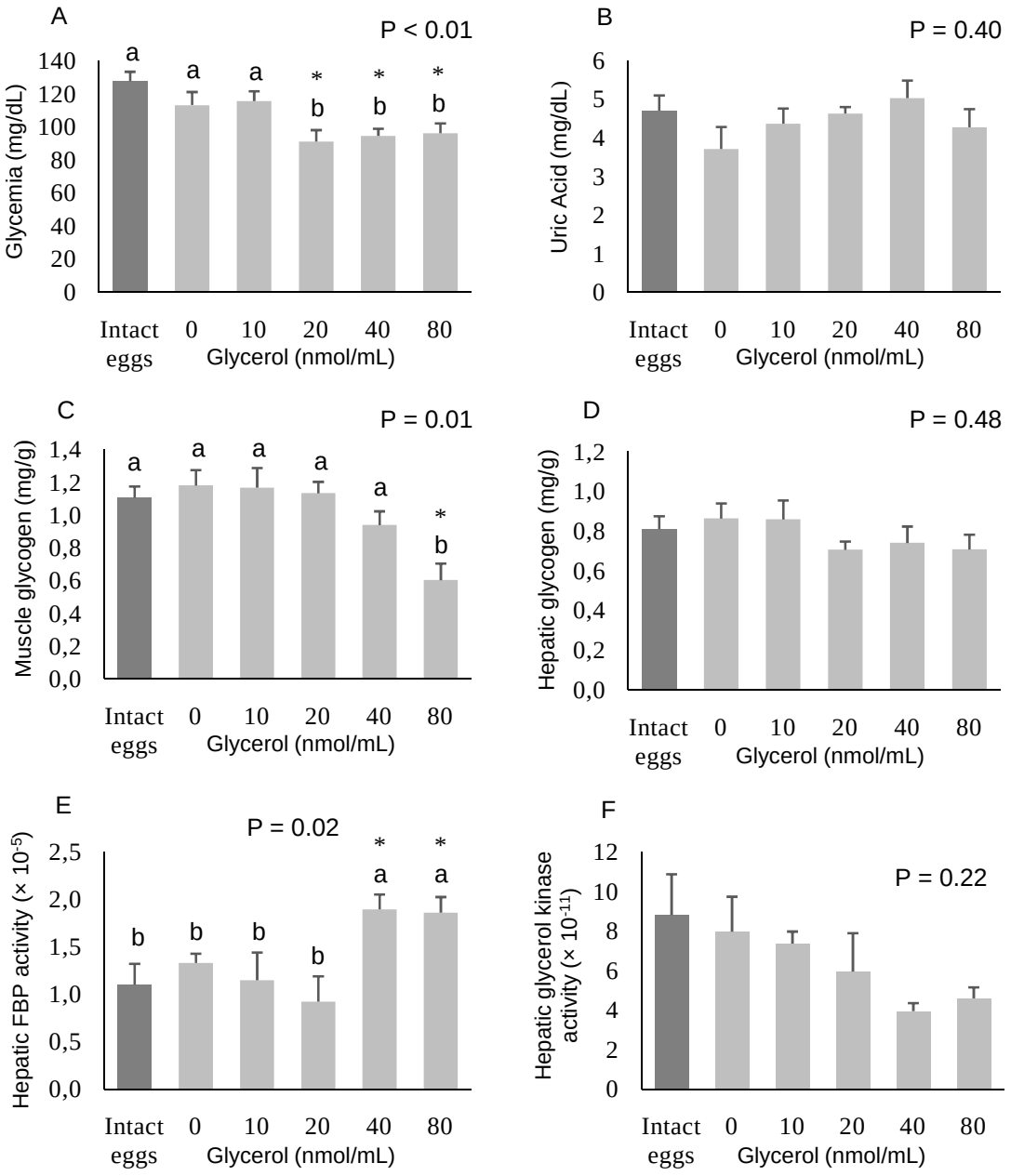
564 **Table 4** Body composition of different aged broilers from non-injected eggs and
565 injected eggs with solutions containing different concentrations of glycerol.

Variable	Non-injected	Saline solution	Glycerol (nmol/mL)				SEM	P value
			10	20	40	80		
1 day of age								
Live weight (g)	49.4	52.4	52.9	51.1	51.9	52.4	-	-
Heart (%)	0.89	0.83	0.82	0.80	0.82	0.76	0.016	0.27
Liver (%)	2.08	2.08	1.93	2.05	1.93	2.03	0.032	0.58
Gizzard (%)	3.91	3.94	3.89	4.26	3.77	3.89	0.104	0.72
Yolk residue (%)	17.66	17.07	17.70	16.87	17.91	17.75	0.376	0.92
Breast (%)	3.82	3.69	3.80	4.01	3.92	3.86	0.072	0.78
Thigh + drumstick (%)	6.07	6.10	6.72	6.56	6.22	5.91	0.120	0.23
Small intestine (%)	2.92	3.06	3.04	3.40	3.02	2.61	0.088	0.08
Duodenum (%)	0.49 b	0.49 b	0.52 a	0.57 a	0.56 a	0.42 b	0.002	0.01
Jejunum (%)	0.58	0.55	0.58	0.64	0.58	0.52	0.640	0.20
Ileum (%)	0.61	0.49	0.54	0.58	0.55	0.48	0.256	0.30
Large intestine (%)	1.40	1.48	1.33	1.55	1.36	1.14	0.048	0.18
7 days of age								
Live weight (g)	134	106	146	145	136	117	-	-
Heart (%)	1.02	1.16	1.03	0.92	0.97	1.02	0.003	0.06
Liver (%)	3.22	3.45	3.35	3.31	3.10	3.14	0.038	0.56
Gizzard (%)	6.74	6.85	6.01	6.41	6.74	7.20	0.144	0.15
Yolk residue (%)	0.25	2.45	1.43	0.71	0.41	1.51	0.069	0.45
Breast (%)	15.0 a	13.0 b	16.3 a	15.8 a	15.2 a	14.7 a	0.650	0.02
Thigh + drumstick (%)	7.48	7.47	7.54	7.37	7.24	7.84	0.112	0.41
Small intestine (%)	8.00	10.83	9.46	9.54	9.16	9.99	0.633	0.39
Duodenum (%)	1.60 b	1.74 b	1.66 b	1.91 a	1.97 a	1.82 a	0.012	0.04
Jejunum (%)	2.38	2.37	2.44	2.76	2.64	2.22	0.025	0.12
Ileum (%)	1.69	2.02	1.85	2.02	1.97	1.90	0.019	0.56
Large intestine (%)	2.33	4.70	3.51	2.85	2.58	4.04	0.222	0.40
40 days of age								
Live weight (g)	2012	2138	2266	2206	2163	2251	-	-
Heart (%)	0.46	0.47	0.42	0.46	0.43	0.41	0.012	0.59
Liver (%)	1.79	1.86	1.76	1.71	1.76	1.78	0.037	0.79
Gizzard (%)	2.03	2.39	2.11	2.28	2.34	2.09	0.070	0.36
Carcass (%)	75.82	76.41	79.35	78.63	79.64	80.05	0.895	0.42
Breast (%)	23.62	24.28	25.05	24.97	25.03	25.90	0.507	0.74
Thigh + drumstick (%)	17.59	18.85	18.78	18.08	18.91	18.47	0.293	0.47
Small intestine (%)	3.44	4.08	3.92	4.00	3.62	3.61	0.119	0.30
Duodenum (%)	0.49	0.61	0.51	0.63	0.57	0.53	0.019	0.16
Jejunum (%)	1.04	1.23	1.17	1.19	1.10	1.19	0.049	0.75
Ileum (%)	1.26	1.26	1.05	1.11	1.07	1.13	0.040	0.42
Large intestine (%)	1.05	0.98	1.19	1.06	0.87	0.76	0.060	0.22
Abdominal fat (%)	0.72 a	0.78 a	1.12 b	1.16 b	1.24 b	1.09 b	0.064	0.02

566 SEM: standard error of the mean
567 [a.b]Means followed by different letters with in a row differ by a Scott–Knott test (P<0.05)
568 * Diferem pelo teste de Dunnett (P<0.05)



569 **Figure 1.** Hatch window (in hours) of hatching (A) and hatchability (B) of non-injected
570 (intact eggs) and injected eggs with solutions containing different concentrations of
571 glycerol. [A.B] Means followed by different letters differ by a Kruskal Wallis (P<0.01).



572 **Figure 2.** Biochemical characteristics (mean ± standard error) of hatching broilers
573 from non-injected eggs (intact eggs) eggs or inoculated with solutions containing
574 different concentrations of glycerol. [a. b] Different letters indicate difference by the
575 Scott-Knott test (P<0.05). * Differs from control (intact eggs) by the Dunnett test
576 (P<0.05). FBP: Fructose 1.6-biphosphatase.

ARTIGO 2 - Effects of *in ovo* injection of insulin-like growth factor (IGF-I) on development of broiler chickens

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Short title: *In ovo* injection of IGF-I for broilers

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Abstract

The addition of growth factors to fertile eggs may be an innovative methodology to stimulate the embryonic development of birds. The objective of this study was to evaluate the effect of *in ovo* injection of IGF-I on the biochemical parameters, hatchability, intestinal morphometry, performance and carcass characteristics of broilers. A total of 360 fertile eggs were distributed in six experimental groups consisting of five saline solutions (0.9%) containing 0, 12.5, 25, 37.5 or 50 ng/embryo of IGF-I, plus a control group (intact eggs). The injections were performed on the 17th day of incubation. The birds were slaughtered at 1, 7 and 40 days of age. The injection of 50 ng IGF-I/embryo increased (P<0.05) the hatching percentage in relation to the control group. No effect (P>0.05) was observed on incubation time. Eggs that were injected with 12.5 ng or more of IGF-1/embryo hatched chicks with lower glycemia (P<0.05) and higher fructose 1,6-biphosphate phosphatase activity. The dose of 50 ng/embryo increased (P<0.05) the muscle glycogen of birds at 1 day old. At 7 days old, the doses of 25, 37.5 and 50 ng IGF-I/embryo increased (P<0.05) the breast yield. The doses of 12.5, 25 and 37.5 ng IGF-I/embryo increased ($P<0.05$) villus height/crypt depth ratio in the duodenum at hatch and at 7 days old with the injection of 37.5 e 50 ng IGF-I/embryo in the jejunum and with the different concentrations in the ileum. No effect (P>0.05) injection of IGF-I was observed on the blood uric acid content nor on the performance or carcass characteristics in birds at 1 and 40 days of age. It is concluded that 50 ng IGF-I/embryo can be used as a substrate in *in ovo* injection of broilers to increase the hatchability and muscle glycogen content of hatched birds, without influencing the post-hatch performance and carcass characteristics at 40 days old.

Keywords: embryo, fructose 1,6-bisphosphate phosphatase, *Gallus gallus domesticus*, glycogen, *in ovo* nutrition

Implications

Currently, the *in ovo* feeding technique has been indicated to improve the post-hatch performance of broilers, among other advantages. However, the major problem with this method is the reduction of hatchability. In the present study, we observed that *in ovo* injection with IGF-I increased hatchability and improved some metabolic aspects of hatching broilers. Although no effects on the performance and carcass characteristics were observed, these findings represent a basis for future studies using growth factors, isolated or associated with nutrients, in the *in ovo* feeding for birds.

Introduction

In the last decades, different nutritional programs have been tested in order to improve the performance and carcass characteristics of broiler birds. Among the different techniques, *in ovo* feeding has been highlighted (Retes *et al.*, 2018). Together with the use of the *in ovo* vaccination method, the possibility of inoculating eggs with different substances that have the potential to promote embryo development has been evaluated.

IGF-I has known effects in both mammals and birds. During the embryonic phase, IGF-I can be found in the amniotic fluid. Its function is probably related in the regulation of the use of amino acids by the embryo (Karcher *et al.*, 2005). Studies have also shown that IGF-I is capable of stimulating hepatic glycogen synthesis, and RNA and protein synthesis in the muscle (McMurtry, 1998, Lu *et al.*, 2007), in

addition to having an important role in the differentiation and growth of muscle fibers (Kocamis *et al.*, 1998, Liu *et al.*, 2011), directly influencing body development. In fact, Liu *et al.* (2011) observed that the injection of 100 ng of recombinant human IGF-I in eggs on the 12th day of incubation stimulated the development of muscle mass in turkeys. In addition, IGF-I has an important relationship with the development of the intestinal mucosa (Foye *et al.*, 2006). In this case, the improvement of the absorptive capacity of the intestine could reduce the permanency of nutrients in the gastrointestinal tract and, consequently, reduce problems with diarrhea and improve the performance of the birds, while reducing the emission of pollutants by excreta.

During incubation, IGF-I levels are elevated, but they decrease during hatching and increase rapidly thereafter (Lu *et al.*, 2007). Until now, no study has evaluated the injection of IGF-I in embryonated broiler eggs during the final stage of embryonic development. The hypothesis is that the injection of this substance can improve the performance of hatching birds and carcass characteristics by favoring muscle development before hatching. Therefore, the objective of this study was to evaluate the effect of injection IGF-I on the hatchability, biochemical parameters post-hatch, intestinal morphometry, performance and carcass characteristics of broiler chickens.

Material and methods

Incubation

The experiment was conducted in the Poultry Sector of the Department of Animal Science of the Federal University of Lavras (UFLA) in Lavras, Minas Gerais, Brazil. The methodology was approved by the Institutional Animal Care and Use Committee under protocol number 49/14.

A total of 480 fertile eggs (weight 70.4 ± 3.9 g) from 55-week-old Cobb 500® hens were purchased from a commercial hatchery. Initially, the eggs were pre-heated to 30 °C for 12 hours, fumigated with 37% formaldehyde and potassium permanganate (2:1) and then distributed randomly in incubator trays (Luna 480, Chocmaster, Piraquara, Brazil), with an automatic adjustment of temperature and humidity. Throughout the incubation period, the temperature remained at 37.4 ± 0.5 °C and humidity remained at 60 ± 0.9% (Cobb, 2013). On the 17th day of incubation, the eggs were evaluated under light to remove the infertile eggs, those with early mortality (small embryos in relation to age) and those with malformed air chambers. In total, 60 fertile eggs were used for each experimental group.

Inoculated solutions

The experimental groups consisted of five 0.9% saline solutions with different concentrations of IGF-I (human insulin-like growth factor-I, Sigma Aldrich, Darmstadt, Germany; (Kocamis *et al.*, 1998): 0, 12.5, 25.0, 37.5 and 50.0 ng/embryo, plus a control group (intact eggs), totaling six experimental groups. The pH and osmolarity of the solutions to be inoculated were evaluated using a pH meter (W3B; Bel Engineering, Monza, Italy) and an osmometer (K-7400, Kanauer, Berlin, Germany) (Table 1).

On the 17th day of incubation, the eggs were weighed and randomly distributed among the different experimental groups. For the inoculated groups, a volume of 0.5 mL of the solutions containing different concentrations of IGF-I was inoculated into the amniotic fluid using a 1-mL disposable syringe and a 22-gauge needle. Prior to injection, the solutions to be inoculated were pre-heated to 30 °C and the eggs were sanitized with 2% iodinated alcohol in the region of needle insertion. After injection,

the hole in the shell was sealed with paraffin (Gonzales *et al.*, 2013). After each injection, the needles were replaced. To ensure that the injection site was the amniotic fluid, 15 embryonated test-eggs were inoculated with a water-soluble dye and then broken to verify the inoculated site.

After injection, the eggs was placed in bride sacs containing the identification of the experimental group (Pedroso *et al.*, 2006). The whole process was carried out in a sanitized room that was maintained at an average temperature of 30 °C. The time that the eggs remained outside the incubator did not exceed 1 hour.

Animal husbandry

The hatching times were registered. After 26 days of incubation, the non-hatched eggs were opened and the embryo mortality period was classified before and after injection by embryodiagnosis (Cobb, 2013). Hatchability was obtained by dividing the number of eggs that hatched with viable chicks by the number of fertile eggs on the 17^{th} day.

Soon after the hatch and drying of the feathers, the birds were housed separately, according to the experimental group, in 2.0 m long × 1.5 m wide boxes with floors covered with shavings, a tubular feeder and an infant-type drinker, which was replaced 7 days later by the pendulum type; the boxes were in a screen masonry shed. On the 22^{nd} day of incubation (48 hours after the first bird had hatched), the birds were weighed, sexed and housed in mixed lots. At this time, the experimental design was in randomized blocks (initial weight and male:female ratio), with six treatments with five replicates of 7 ± 1 birds each.

Before the arrival of the birds, the shed was pre-heated with infrared lamps. The environmental conditions before and after the bird accommodation were monitored

716 through two thermohygrometers (Simpla TH02, Asko, São Leopoldo, Brazil) placed in
717 the shed at the height of the birds. The light program was 24 hours of light. During
718 this phase, all birds received water and feed *ad libitum*; the diet was formulated with
719 corn and soybean meal (Rostagno *et al.*, 2011)(Table 2). Weight gain, feed intake
720 and feed conversion were calculated at the beginning and end of each week. The
721 birds remained in the experimental shed for 40 days.
722
723 *Sample collection*
724 Soon after hatching, six male birds from each experimental group were
725 randomly selected and euthanized by cervical dislocation. Blood sampling was
726 performed during bleeding for the subsequent quantification of plasma glucose and
727 uric acid. After collection, the blood was immediately sent to the laboratory for
728 centrifugation (Sorvall TM ST16 Centrifuge, Thermo Fisher Scientific, Massachusetts,
729 USA) at 1000 x *g* for 15 minutes and stored at -80 °C until further analysis.
730 In sequence, the heart, liver, gizzard, yolk sac, breasts, thigh plus drumstick
731 and small and large intestine were removed and weighed. The liver was immersed in
732 liquid nitrogen for a few seconds and stored at -80 °C until the determination of
733 fructose-1,6-bisphosphato phosphatase activity and the quantification of hepatic
734 glycogen. The segments from the duodenum, jejunum and ileum were flushed with
735 physiological saline and fixed in Bouin solution for 24 h. At 7 days of age, two birds
736 from each box were selected, according to the average weight of the box. These
737 birds were euthanized following the same procedure described for hatching birds.
738 At 40 days of age, two birds with weights close to the box average were
739 selected and slaughtered by cervical dislocation and posterior bleeding. The birds
740 were plucked and eviscerated. The relative weight of the carcass (without legs and

abdominal fat), viscera, breast (skin and bone) and leg (thigh plus drumstick with skin and bone) were calculated in relation to live weight.

Biochemical analyses

Glucose and uric acid were quantified by an enzymatic colorimetric test (Liquiform Glucose, Liquiform Uric Acid, Labtest, Lagoa Santa, Brazil), following the manufacturer's recommendations.

For fructose-1,6-bisphosphato phosphatase activity, the liver (±0.2 g) was homogenized in 1 mL of solution containing 0.1 M Tris-HCl (pH 7.5), 0.15 M KCl, 5 mM dithiothreitol and 5 mM $MgSO_4$. After tissue homogenization, the sample was centrifuged (20600 x *g* for 60 minutes at 4 °C) and the supernatant was collected according to the methodology proposed by Aoki *et al.* (1999). The determination of the enzymatic activity was performed according to the methodology used by Ulm *et al.* (1975).

The hepatic and muscular glycogen contents were determined according to Willems *et al.* (2014). For this, liver and breast fragments (± 0.2 g) were homogenized in 7% perchloric acid solution. After complete homogenization of the tissue, the sample was centrifuged (14000 x *g* for 15 minutes at 4 °C) and the supernatant was collected. Quantification was determined by color reagent based on iodine.

Intestinal Morphometry

Samples of the duodenum, jejunum and ileum with approximately 5 mm in length were subjected to routine histological procedures, being dehydrated in solutions with increasing concentrations of ethanol, cleared in xylene, embedded in

paraffin, microtomized to 4.0 µm, arranged on silanized glass slides, dried at 37 °C overnight and stained with haematoxylin and eosin (Suvarna et al.*, 2018). Two cuts of different regions were performed for each intestinal segment, of which scanned images were analysed using an Olympus microscope (CX31; Olympus, Tokyo, Japan) coupled to an Altra SC30 digital camera (Olympus) using the AxioVision program (Carl Zeiss, Oberkochen, Germany) at 100 x magnification for the duodenum, 200 x for the jejunum and ileum to birds at one day of age and 100 x for the different segments to birds seven days of age. Ten readings were taken per slide, measuring villus height (VH) and crypt depth (CD). VH was measured from the tip of the villi to the villus crypt junction and CD was defined as the depth of the invagination between adjacent villi. The average value was calculated per variable per chick. Later, villus: crypt ratio was calculated (VH/CD).*

Statistical analysis

The hatchability data were submitted to a binomial model and the main effect (treatments) was analyzed by a likelihood ratio test. Means were compared by a Pearson Chi-square test (Leitão *et al.*, 2010). The other data were submitted to tests for normality (Shapiro–Wilk), homoscedasticity of variance (Breusch–Pagan) and independence of errors (Durbin–Watson); in the case of non-significance on these tests, an analysis of variance (ANOVA) was performed and IGF-I levels were submitted to a regression analysis. The means obtained with each level of IGF-I were compared to the control (intact eggs) by a Dunnett's test. When there was no adjustment of the regression curve (R_2<0.70), the means were compared by a Scott–Knott test at 5%. For the variables that did not meet the ANOVA assumptions, the Box-Cox data transformation option was used. The variables that did not reach

normality, even after data transformation, were submitted to non-parametric analysis and the means were compared by a Kruskal–Walis test. All statistical analyses were performed in the statistical program Action version 3.4 (Estatcamp, São Carlos-SP, Brazil).

Results

The injection of the solution with 50 ng IGF-I/embryo increased hatchability, in relation to the intact eggs (P=0.05; Table 3). There was no influence of the different inoculated solutions (P>0.05) on the incubation time.

The injection of solutions containing at least 12.5 ng/embryo reduced (P<0.01) glycemia and increased (P<0.01) the glycogen content in the breast muscle of birds at 1 day of age (Table 4). *In ovo* injection of the solutions containing different concentrations of IGF-I increased (P=0.05) the hepatic glycogen content, in relation to the intact eggs, and also increased the activity of fructose 1,6-biphosphate phosphatase in the liver. There was no effect ($P>0.05$) of the injection of the different solutions on the concentration of uric acid in the blood.

The 7-day-old birds had a higher relative breast weight (P<0.05) when they were inoculated with solutions containing 25, 37.5 and 50 ng IGF-I/embryo, in relation to intact eggs (Table 5). There was no effect (P>0.05) of the injection of the different solutions on the carcass characteristics of birds at 1 or 40 days of age. There was also no effect (P>0.05) on the performance of the birds at 7, 14, 21 and 39 days of age (Table 6).

Higher villus height and depth of crypt in the duodenum were observed ($P<0.05$) in birds from intact eggs and the best villus height/crypt depth ratio was obtained ($P<0.01$) with injection of 12.5, 25 and 37.5 ng IGF-I/embryo at 1 day of age

(Table 7). The injection with 37.5 e 50 ng IGF-I/embryo decreased (*P=0.02*) crypt depth in the jejunum at this age. At 7 days of age, in ovo injection of the solutions containing 37.5 e 50 ng IGF-I/embryo increased (*P<0.01*) villus height/crypt depth ratio in the jejunum and the different concentrations of IGF-I also increased (P<0.01) this ratio in the ileum.

Discussion

IGF-I is a growth factor with well-known effects in the body. In the literature, evidence proves the role of this hormone in the embryo development of broilers (Dishon *et al.*, 2018), but few have verified the influence of *in ovo* feeding of this substance on post-hatch development (Kocamis *et al.*, 1998, Mohammadrezaei *et al.*, 2015). In the present study, the most evident effects of *in ovo* injection of IGF-I were an increase in hatchability and the deposition of muscular glycogen. There were no significant effects on broiler performance or carcass characteristics post-hatch.

In recent years, most studies have reported that the *in ovo* feeding technique, regardless of the substance used, reduces hatchability compared to intact eggs (Salmanzadeh *et al.*, 2012, Lotfi *et al.*, 2013). However, this result can be attributed, among other causes, to the injection technique, which is still not well defined (Retes *et al.*, 2018). On the other hand, some experiments show that *in ovo* feeding nutrients may have a positive influence on hatchability (Hassan *et al.*, 2018, Khaligh *et al.*, 2018). The present study is the first to show the positive effect of *in ovo* injection of IGF-I at 17 days of incubation on hatchability in broilers.

In a commercial hatchery, increasing the number of hatched eggs is always economically desirable. In the present study, injection of a solution containing 50 ng/embryo or 25 ng/egg increased hatchability by 14%, compared to the non-

inoculated group. Kocamis *et al.* (1998)observed an average reduction of 17% when they inoculated 100 ng/egg of this same substance on days 1, 2, 3 and 4 of incubation. The results found in this study, however, may be related to the *in ovo* injection technique, since in the control group (saline), there was also a similar proportion reduction in hatchability. Kocamis *et al.* (1998) used a solution containing acetic acid (10 mM) and bovine serum albumin (BSA; 0.1 g) as diluent, which may have hindered hatchability. In the present study, the use of 0.9% saline did not affect hatchability.

According to Duclos *et al.* (1991), IGF-I can stimulate the expression of proteins in the muscle cells of broilers. These results suggest that this substance plays an important role in embryonic development. In fact, the injection of 100 ng IGF-I/egg between days 7 and 13 of incubation increased the number and diameter of muscle fibers in Japanese quails (Deprem and Gulmez, 2007)and stimulated muscle growth in broilers when they were inoculated on the 3rd day of incubation (Kocamis *et al.*, 1998). In the present study, although a higher breast yield was not observed in birds at 1 day old, greater muscular glycogen deposition was observed. This result demonstrates the effect of this hormone on the muscle characteristics of the birds. In addition, the injection of 25 ng IGF-I/egg on the 17^{th} day of incubation may not have been a large enough dose or did not allow for enough time to increase the relative weight of the birds' chest at 1 day of age; however, this result was evident in birds at 7 days of age. This result may be related to the greater development of muscle fibers in birds from eggs inoculated with IGF-I.

The further development of muscle fibers may be related to an increased metabolic rate. In the present study, the reduction of glycemia during hatching suggests that the increase in the embryonic metabolic rate occurred in the moments

before hatching. In the final stages of incubation, the embryo performs glucose catabolism (De Oliveira *et al.*, 2008) due to a high energy demand for bark pecking and low oxygen tension (Moran Jr., 2007). In this case, the supposed increase in embryo muscle development, associated with the increased metabolic rate due to IGF-I injection (McMurtry, 1998), may be related to increased hatchability and decreased blood glucose at hatching.

Although hatchability was influenced by *in ovo* injection of IGF-I, no differences in hatching time were observed in the present study. An increase in the metabolic rate at the end of the incubation period is usually accompanied by an increase in the respiration rate, resulting in a decrease in O_2 supply and an increase in CO_2 concentrations within the egg (Khaligh *et al.*, 2018). This increase of CO_2 in the egg during the final period of embryo development can accelerate hatching and, consequently, reduce hatching time (Everaert *et al.*, 2007). In the present study, the probable increase in metabolic rate, suggested by the increased glucose uptake by birds from eggs inoculated with IGF-I, was not sufficient to reduce the hatching time. On the other hand, the predominance of anaerobic metabolism, which occurs before hatching (De Oliveira *et al.*, 2008), may have limited the use of O_2 for energy metabolism and, consequently, avoided the elevation of CO_2; this could be responsible for the reduction in hatching time. In a hatchery, a reduction of hatching time is economically feasible, considering the time the eggs remain in the incubator. However, when eggs hatch too early, chicks become susceptible to problems such as dehydration, which may lead to increased mortality up to the second week of life or low performance chickens (Cobb, 2013). In this case, IGF-I can be safely used to increase hatchability without affecting hatching time.

During embryonic development, the plasma IGF-I level increases from day 6 to a peak at day 15 of incubation, then declines to low levels at hatching (Lu *et al.*, 2007). This decline may be related to the targeting of energy metabolism to glycogen as a source of energy (McMurtry *et al.*, 1997). In fact, studies show that IGF-I can stimulate hepatic glycogen synthesis (McMurtry, 1998, Lu *et al.*, 2007). In the present study, only the muscle glycogen content was influenced by the injection of IGF-I; the dose of 50 ng/embryo was the only dose that resulted in this increase. IGF-I did not appear to influence post-hatching hepatic gylcogen, since the values were statistically similar to those of the eggs that were inoculated with saline solution. On the other hand, as reported by Khaligh *et al.* (2018), the increase in anaerobic metabolism during the final incubation stage may be associated with a greater dependence on glucose, whose source may be hepatic glycogen. Before hatching, glycogenolysis supports the hatching process in response to energetic demands from glycogen (Picardo and Dickson, 1982). At that time, the elevation of glucagon levels would be one of the factors responsible for hepatic glycogenolysis (Lu *et al.*, 2007). Therefore, in the present study, the fact that the hepatic glycogen concentration was similar to that of the saline solution does not rule out the possibility that IGF-I may have stimulated the deposition of this carbohydrate in the liver in the moments before hatching. In fact, Parkes *et al.* (1986) verified an increase in the *in vitro* deposition of glycogen in the embryonic cells of birds. The *In vivo* quantification of hepatic glycogen before hatching in eggs inoculated with IGF-I has not been evaluated until the present study.

With regard to the enzymatic activity evaluated in hatching birds, there was an increase in fructose 1,6-bisphosphate phosphatase activity in birds from eggs inoculated with IGF-I. The main role of this enzyme is related to hepatic

gluconeogenesis (Chaekal *et al.*, 1983). At the end of incubation, the high energy demand associated with low O_2 tension requires the embryo to perform anaerobic metabolism, whose main substrate is glucose (De Oliveira *et al.*, 2008). In this case, gluconeogenesis in the liver depends on gluconeogenic nutrients such as lactate, glycerol and amino acids (Watford *et al.*, 1981), reducing the contribution of the latter to protein metabolism. However, the results of the uric acid content in the blood suggest that there was no increase in amino acid catabolism (Tinker *et al.*, 1986). On the other hand, Watford *et al.* (1981) suggested that lactate is one of the main substrates involved in the synthesis of glucose in the liver. Thus, the increased activity of fructose 1,6-biphosphate phosphatase in the liver and a lack of effects on plasma uric acid suggest that the supposed increase in metabolic activity provided by IGF-I stimulated gluconeogenesis, but not from amino acids. As the glycemia of hatching birds from eggs inoculated with IGF-I decreased, it is proposed that lactate may have been the main gluconeogenic precursor at the end of incubation.

Although IGF-I positively influenced the embryonic metabolism of the birds, influences on post-hatch performance and carcass characteristics were not observed, except for the breast weight of 7-day-old birds, whose values were higher when 25, 37.5 or 50 ng/embryo were used. Girbau *et al.* (1987) observed positive effects on the organogenesis of birds with the injection of 100 ng of IGF-I on the second day of incubation. Similar results to those of the present study, related to viscera weight, were also observed by Spencer *et al.* (1990) when inoculating birds with 500 ng of IGF-I at both 7 and 14 days of incubation. On the other hand, Liu *et al.* (2011), while studying the embryonic development of ducks from eggs inoculated with 100 ng of IGF-I at 12 days of incubation, observed a higher weight of the embryos at 27 days of incubation and in the birds 2 days after hatching. In this case,

in addition to the species being different (lower genetic potential), the time between injection and the observation of the results was different than the time period evaluated in the present study. Although the relative breast weight at 7 days was higher when 50 ng IGF-I/embryo was inoculated into the egg, this increase was not enough to influence the performance of the bird in the first week of life.

An increase in the post-hatch morphological differentiation of the small intestine has been reported after the injection of growth factors in the amniotic fluid during incubation in duck eggs (Wang *et al.*, 2012). According to the authors, IGF-1 plays an important role in cell proliferation and organ formation through different physiological forms. In the present study, IGF-I injection did influence the relative weight of the organs. However, it promoted greater in the development of the intestinal epithelium. A high villus height is associated with a well differentiated intestinal mucosa with high digestive and absorptive capabilities (Jeurissen *et al.*, 2002). Furthermore, a deeper crypt is indicative of a faster tissue turnover (Berrocoso *et al.*, 2017). Thus, villus height, crypt depth and villus length/depth ratio are considered good indicators of functional capacity of the intestine (Fasina and Olowo, 2013). Despite the improvement in intestinal epithelial development, especially jejunum, as measured by villus height /depth ratio, IGF-I injection had no effect in performance post-hatch.

Kocamis *et al.* (1998) observed a greater weight gain and better feed conversion in broilers at 42 days of age when they had been inoculated with 100 ng of IGF-I in the eggs on the third day of incubation Mohammadrezaei *et al.* (2015) also reported a higher weight gain and lower feed conversion at 42 days in broilers after they were inoculated with 100 or 200 ng of IGF-I on the fifth day of egg incubation. In the present study, since there was no effect of IGF-I on the performance and carcass characteristics after hatching, it is believed that the injection of IGF-I on the 17th day

of incubation was late, compared to the other studies. However, since *in ovo* vaccination is a technique that is normally practiced at this time in commercial hatcheries, the association of growth factors or nutrients capable of improving the performance of hatching birds is advantageous. From the results of the present study, it is believed that the use of IGF-I at a dose of 50 ng/embryo or greater, associated with the vaccine programs, can produce positive results. However, studies of the association of this hormone with other substances that influence the embryonic metabolism of birds are important to verify the viability of using this technique at different incubation times.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

Ethics statement

The protocol applied to animal experiments was approved (protocol number 49/14) by the Institutional Animal Care and Use Committee of Federal University of Lavras (Lavras, Minas Gerais, Brazil).

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1111 2014. Embryonic protein undernutrition by albumen removal programs the hepatic
1112 amino acid and glucose metabolism during the perinatal period in an avian model.
1113 PLoS One 9, e94902.

1114 **Table 1** pH and osmolarity of the used solutions.

Solution	pH	Osmolarity (mOsm)
0.9% saline solution	7.00	324
25 ng/mL of IGF-I in 0.9% saline solution	7.42	313
50 ng/mL of IGF-I in 0.9% saline solution	7.44	314
75 ng/mL of IGF-I in 0.9% saline solution	7.49	312
100 ng/mL of IGF-I in 0.9% saline solution	7.56	330

Table 2 Centesimal composition and calculated nutritional levels of the experimental diets used during the different stages of the post-hatch development of broilers.

Ingredient	1-7 days	8-21 days	22-35 days	36- 40 days
Corn	55.489	59.645	62.377	67.093
Soybean meal (45%)	38.162	34.664	31.437	27.218
Soy oil	2.055	2.013	2.963	2.787
Dicalcium phosphate	1.907	1.508	1.273	1.070
Calcitic limestone	0.914	0.924	0.865	0.771
Salt	0.507	0.482	0.457	0.444
DL-Methionine (99%)	0.359	0.287	0.256	0.240
L-Lysine (78%)	0.289	0.219	0.196	0.237
L-Threonine (99%)	0.114	0.064	0.043	0.053
Salinomycin	0.050	0.050	0.050	0.000
Vitamin supplement [1]	0.050	0.040	0.030	0.020
Mineral supplement [2]	0.050	0.050	0.050	0.050
Choline chloride (60%)	0.050	0.050	0.000	0.020
Avilamycin	0.005	0.005	0.005	0.000
TOTAL	100.00	100.00	100.00	100.00
Calculated nutritional composition				
Metabolizable energy, kcal/kg	2950	3000	3100	3150
Crude protein, %	22.20	20.80	19.50	18.00
Calcium, %	0.920	0.819	0.732	0.638
Available phosphorus, %	0.470	0.391	0.342	0.298
Digestible lysine, %	1.310	1.174	1.078	1.010
Digestible methionine + Cystine, %	0.944	0.846	0.787	0.737
Digestible threonine, %	0.852	0.763	0.701	0.656
Sodium, %	0.220	0.210	0.200	0.195

1. Supply per kg of product: 5.000.000 IUof vitamin A; 1.850.000IUof vitamin D3; 4.500 IUof vitamin E; 918 mg of vitamin K3; 2.000 mg of vitamin B2; 250 mg of vitamin B6; 6,500 mcg of vitamin B12; 145.4 mg of folic acid; 12.9 g of niacin; 5,931 mg of pantothenic acid and 480 mg of selenium.

2. Supply per kg of product: 67.5 g of manganese; 50.4 g of iron; 43.2 g of zinc; 7.0 g of cupper and 1,464 mg of iodine.

Table 3 Hatchability and incubation time of intact eggs and eggs inoculated with solutions containing different concentrations of IGF-I.

Variable	Intact eggs	Saline solution	IGF-I (ng/embryo)				P value
			12.5	25.0	37.5	50.0	
Hatchability (%)	82.1 b	81.5 b	79.7 b	72.0 c	89.3 ab	93.9 a	0.05
Incubation time (hours)	521	523	518	527	519	523	0.17

[a,b]Means followed by different letters within a row differ by a Pearson Chi-square test (P<0.05).

Table 4 Biochemical parameters in 1-day-old broilers from intact eggs and eggs inoculated with solutions containing different concentrations of IGF-I.

Variable	Intact eggs	Saline solution	IGF-I (ng/embryo)				CV (%)	P value
			12.5	25.0	37.5	50.0		
Blood glucose (mg/dL)	120.6 a	112.2 a	90.1 b	81.4 b	91.7 b	101.2 b	16.08	<0.01
Plasma uric acid (mg/dL)	3.56	4.07	3.59	3.76	4.02	4.06	16.99	0.65
Hepatic glycogen (mg/g)	4.94 a	6.86 b	7.55 b	6.46 b	6.96 b	6.17 b	27.49	0.05
Muscle glycogen (mg/g)	2.91 a	2.78 a	3.18 a	3.27 a	3.03ab	4.31 b	22.09	<0.01
Hepatic fructose 1,6-biphosphate phosphatase activity($\times 10^{-6}$)	1.79 a	2.38 a	5.04 b	4.82 b	3.72 b	3.49 b	19.06	<0.01

CV: coefficient of variation

[a,b]Means followed by different letters within a row differ by a Scott–Knott test (P<0.05)

Table 5 Carcass composition of different aged broilers from intact eggs and eggs inoculated with solutions containing different concentrations of IGF-I.

Variable	Intact eggs	Saline solution	IGF-I (ng/embryo)				CV (%)	P value
			12.5	25.0	37.5	50.0		
1 day of age								
Live weight (g)	53.3	51.4	52.1	52.9	52.9	51.7	-	-
Heart (%)	0.71	0.74	0.76	0.69	0.73	0.65	16.9	0.55
Liver (%)	1.93	2.04	1.94	1.90	1.87	1.79	10.8	0.30
Gizzard (%)	3.90	3.80	4.27	4.21	4.17	3.56	14.9	0.13
Yolk residue (%)	17.2	17.9	15.9	18.9	17.3	20.4	22.4	0.34
Breast (%)	4.02	3.61	4.05	3.99	3.62	3.85	12.9	0.25
Thigh + drumstick (%)	5.80	5.64	6.02	5.71	5.87	5.92	12.7	0.92
Small intestine (%)	3.07	3.21	3.17	3.06	2.91	2.79	19.9	0.72
Duodenum (%)	0.51	0.50	0.50	0.46	0.47	0.43	20.3	0.59
Jejunum (%)	0.64	0.58	0.61	0.62	0.57	0.54	16.9	0.48
Ileum (%)	0.58	0.56	0.59	0.59	0.57	0.55	21.2	0.96
Large intestine (%)	1.34	1.57	1.48	1.38	1.30	1.27	32.7	0.77
7 days of age								
Live weight (g)	123.0	123.4	140.9	119.9	137.9	124.4	-	-
Heart (%)	0.99	1.01	0.92	1.00	0.97	0.99	11.35	0.52
Liver (%)	3.44	3.31	3.30	3.27	3.24	3.42	9.91	0.69
Gizzard (%)	6.58	6.33	5.96	6.87	6.40	6.45	12.08	0.21
Yolk residue (%)	0.29	0.19	0.21	0.45	0.31	0.43	-	0.76
Breast (%)	14.7 b	14.7 b	13.8 b	15.6 a	16.0 a	15.4 a	9.93	0.04
Thigh + drumstick (%)	7.38	7.53	7.64	7.69	7.54	7.52	4.48	0.39
Small intestine (%)	8.70	8.08	7.51	8.46	7.96	8.31	12.26	0.14
Duodenum (%)	1.72	1.60	1.51	1.52	1.72	1.71	12.58	0.15
Jejunum (%)	2.37	2.33	2.18	2.49	2.30	2.28	13.41	0.35
Ileum (%)	1.89	1.81	1.60	1.78	1.77	1.73	16.13	0.34
Large intestine (%)	2.71	2.34	2.21	2.46	2.37	2.58	24.53	0.49
40 days of age								
Live weight (g)	2504	2669	2621	2618	2700	2603	-	-
Heart (%)	0.47	0.45	0.45	0.44	0.44	0.43	12.6	0.86
Liver (%)	1.79	1.62	1.78	1.64	1.70	1.80	8.8	0.12
Gizzard (%)	2.10	1.95	1.88	2.08	1.90	1.94	19.2	0.83
Carcass (%)	80.6	80.3	80.4	79.7	81.4	80.3	2.4	0.81
Breast (%)	26.2	26.3	27.1	24.3	26.6	26.2	9.6	0.63
Thigh + drumstick (%)	19.6	19.2	19.9	20.3	20.6	20.0	7.6	0.61
Small intestine (%)	3.88	3.68	3.74	3.91	3.61	3.70	14.5	0.91
Duodenum (%)	0.57	0.52	0.57	0.55	0.56	0.57	19.0	0.93
Jejunum (%)	1.17	1.07	1.11	1.06	0.98	1.10	19.7	0.64
Ileum (%)	1.13	1.06	1.06	1.04	1.06	1.03	18.6	0.92
Large intestine (%)	1.00	1.03	1.01	1.20	1.01	1.01	19.3	0.68
Abdominal fat (%)	1.07	1.07	0.83	0.86	1.05	1.08	33.2	0.54

CV: coefficient of variation

Table 6 Performance of broilers from intact eggs and eggs inoculated with solutions containing different concentrations of IGF-I.

Performance	Intact eggs	Saline solution	IGF-I (ng/embryo)				CV (%)	P value
			12.5	25.0	37.5	50.0		
1 to 7 days of age								
Feed intake (g)	92.5	97.5	91.8	106.5	110.0	97.0	10.1	0.14
Weight gain (g)	91.1	94.5	96.9	84.6	96.6	90.8	7.3	0.17
Feed conversion	1.02	1.03	1.10	1.10	1.14	1.07	9.7	0.48
1 to 14 days of age								
Feed intake (g)	489	502	518	478	535	505	8.0	0.29
Weight gain (g)	445	455	437	420	430	421	5.4	0.16
Feed conversion	1.10	1.11	1.19	1.15	1.25	1.20	8.8	0.20
1 to 21 days of age								
Feed intake (g)	1117	1166	1147	1155	1156	1102	6.2	0.67
Weight gain (g)	858	868	853	877	864	788	6.5	0.18
Feed conversion	1.30	1.34	1.35	1.32	1.34	1.40	5.8	0.52
1 to 39 days of age								
Feed intake (g)	3669	3783	3747	3574	3749	3753	7.3	0.83
Weight gain (g)	2532	2549	2512	2452	2445	2483	6.7	0.89
Feed conversion	1.45	1.49	1.49	1.46	1.54	1.51	5.2	0.52

CV: coefficient of variation

Table 7 Intestinal epithelium morphometry of different aged broilers from Intact eggs eggs and inoculated with solutions containing different concentrations of IGF-I.

Variable	Intact eggs	Saline solution	IGF-I (ng/embryo)				CV (%)	P value
			12.5	25	37.5	50.0		
1 day of age								
Duodenum								
VH	439 a	353 a	369 b	374 b	420 b	344 b	18.6	0.05
CD	70 a	58 b	47 c	44 c	54 b	57 b	12.6	<0.01
VH/CD	6.4 b	6.1 b	7.9 a	8.6 a	7.9 a	6.1 b	23.9	<0.01
Jejunum								
VH	220	204	207	239	216	253	16.9	0.10
CD	50 a	48 a	48 a	53 a	41 b	43 b	15.3	0.02
VH/CD	4.5	4.3	4.8	4.9	5.4	5.9	24.1	0.11
Ileum								
VH	183	181	185	181	195	193	14.1	0.84
CD	47	39	42	44	43	44	18.0	0.50
VH/CD	4.05	4.76	4.41	4.23	4.63	4.45	19.7	0.62
7 days of age								
Duodenum								
VH	615	650	549	602	557	726	21.7	0.17
CD	102	104	97	98	78	117	21.8	0.08
VH/CD	6.2	6.4	5.8	6.2	7.2	6.3	19.9	0.47
Jejunum								
VH	559	577	537	513	594	577	17.1	0.45
CD	96	96	97	87	84	82	21.0	0.39
VH/CD	6.0 b	6.0 b	5.5 b	6.0 b	7.3 a	7.5 a	20.1	<0.01
Ileum								
VH	416	389	396	423	430	406	16.0	0.72
CD	78	72	67	78	66	67	26.8	0.51
VH/CD	5.5 b	5.5 b	6.0 a	6.1 a	6.7 a	6.4 a	15.6	<0.01

VH: villus height (μm); CD: crypt depth (μm); VH/CD: ratio villus height and crypt depth (μm/μm)

[a,b] Means followed by different letters within a row differ by a Scott–Knott test (P<0.05).

ARTIGO 3 - *In ovo* injection with glycerol and insulin-like growth factor (IGF-I): hatchability, intestinal morphometry, performance and carcass characteristics of broilers

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Abstract

This study was conducted to determine the effects of *in ovo* injection with glycerol (GLY) and IGF-I (IGF) on hatchability, biochemical parameters, intestinal morphometry, performance and carcass characteristics of broiler chickens. A total of 400 fertilized eggs were distributed in five experimental groups. The treatments were arranged as non-injected (control), saline solution injected (0.9% NaCl solution), GLY solution injected (10 nmol/mL), IGF-I solution injected (100 ng/mL), and GLY + IGF-I solution injected. At 17.5 d of incubation, 0.5 mL of each solution was injected into the amniotic fluid of each egg of injected groups. Hatchability and hatch time did not differ (P>0.05) among *in ovo* injection treatments. At hatch, higher (P=0.01) hepatic glycogen was observed in broiler chicks were injected with GLY or IGF-I in comparison to those of control, while higher muscle glycogen was observed (P=0.05) with GLY. The substances increased (P=0.05) the relative weight of the liver and decreased (P<0.05) the fructose 1,6-bisphosfate phosphatase activity. Higher villus height in the jejunum and ileum was observed (P<0.01) with GLY in comparison to those of control. At 7 d of age, lower liver relative weights and higher ileum relative weights were observed (P<0.05), respectively, with GLY and IGF-I. No other carcass characteristics differed (P>0.05) at this age or at 42 d of age. At 14 d of age, the eggs were injected with IGF-I resulted in broiler with a higher (P<0.05) weight gain and a lower feed conversion ratio in comparison to those in control. At 35 and 42 d of age, the use of GLY or IGF-I or GLY + IGF-I increased (P<0.05) feed intake and weight gain, without affecting (P>0.05) feed conversion. It is concluded that GLY and IGF-I or GLY + IGF-I can be used to improve the post-hatch performance of broilers, without affecting hatchability and carcass composition.

Keywords: chicken, *in ovo* nutrition, energy substrate, growth factor, embryo development

1. Introduction

Due to the increasing world population, the need to produce more food is constant. Meat is one of the main foods in human nutrition, so investments in animal production technologies are necessary. Among these technologies, the *in ovo* feeding technique represents an important advance allowing early access to the nutrients by the embryo, which can favor embryonic development and survival of chicks after hatch which improves broiler chicken's performance and weight (Abbasi, et al., 2017; Uni, et al., 2005).

Development and growth of embryos are dependent upon the nutrient deposits in the fertile egg (Yadgary and Uni, 2012). However, due to the high genetic potential of birds, it is believed that the nutrients contained in the egg, especially carbohydrates, may be insufficient and limit the development of birds before and after hatch (Retes, et al., 2018). Moreover, in the period before hatch, the increase in the metabolic rate, due to the high energy demand of the hatch process and the low O_2 supply within the egg (Khaligh, et al., 2017), favor anaerobic metabolism (De Oliveira, et al., 2008). At this time, the embryo uses glucose from glycogen reserves or generated by gluconeogenesis from amino acids, glycerol, and lactate (Watford, et al., 1981). Thus, application of the *in ovo* feeding technique with the injection of substances capable of meeting nutritional requirements during this period could save the use of amino acids and improve hatch and the birds' performance characteristics until the age of slaughter.

It is well documented that glycerol (GLY) can be used as a source of energy for broiler chicken embryos (Neves, et al., 2016). Studies have also reported positive correlation between glycogen stores and body weight at hatch (Christensen, et al., 2000; Tangara, et al., 2010), which is attributed to sparing muscle protein catabolism by energy (Uni et al., 2005). The GLY is an important precursor for gluconeogenesis (Sunny and Bequette, 2011), so *in ovo* feeding of this substance should increase glycogen stores and reduce the use of amino acids as

gluconeogenic compounds to meet the high energy demand for glucose in the final phase of incubation. Thus, GLY injection could increase the amino acids targeting for protein synthesis, favoring not only the development of birds during the hatch process and after hatch, but also birds' performance and carcass characteristics until the age slaughter.

In addition, insulin-like growth factor I (IGF-I) has been shown to play an important role in metabolic control, differentiation and tissue growth (Liu, et al., 2011). During embryogenesis IGF-I can be found in the amniotic fluid, and its function is probably related to the regulation of the use of amino acids by the embryo (Karcher, et al., 2005). The IGF promotes hepatic glycogen synthesis and RNA and protein synthesis in muscle (Lu, et al., 2007), breast muscle hypertrophy (Kocamis, et al., 1998; Liu, et al., 2011), and intestinal mucosa development (Foye, et al., 2006). In ducks, (Liu, et al., 2011) reported that the inoculation of 100 ng of recombinant human IGF-I in eggs on the 12^{th} day of incubation stimulated the development of muscle mass. The effect of *in ovo* injection of IGF-I on the performance and carcass composition of different aged broilers has not been heavily studied (Kocamis, et al., 1998). It is believed that the associated use of GLY and IGF-I may increase glycogen and protein synthesis, influence the metabolism of birds at hatch and favor their later development, improving the performance and carcass characteristics of broiler chickens up to age at slaughter. The associated use of these substances in *in ovo* nutrition has not been reported in the literature. Thus, the objective of this study was to evaluate the effect of *in ovo* injection with GLY and IGF-I, on the biochemical parameters, hatchability, intestinal morphometry, performance and carcass characteristics of broilers.

2. Material and methods

2.1 Incubation

This experiment was conducted in the Poultry Sector of the Department of Animal Science of the Federal University of Lavras (UFLA) in Lavras, Minas Gerais, Brazil. The protocol was approved by the Institutional Animal Care and Use Committee under number 49/14.

Fertilized Cobb 500® broiler eggs collected from 50 wk hens were obtained from the commercial hatchery. All eggs were selected and weighed individually with an average weight of 69.0 ± 4.1 g. Initially, the eggs were pre-heated to 30 °C for 12 hours, disinfected with 37% formaldehyde and potassium permanganate (2:1) and were randomly distributed in the six incubator tray levels (Luna 480, Chocmaster, Piraquara, Brazil) and incubated at 37.5 ± 0.2 °C and relative humidity at 60 ± 1.7%, so that in all trays contained all the experimental groups. The eggs were candled on 17 d of incubation for selecting embryonated eggs, the unfertilized or nonviable eggs were removed. After candling, a total of 400 embryonated eggs were distributed to 5 experimental groups of 80 eggs in a completely randomized design.

2.2 Inoculated Solutions

The experimental groups were: 1) non-injected (control group), 2) saline solution (0.9% NaCl solution, diluent-injected control group), 3) GLY solution-injected group:10 nmol/mL of GLY dissolved in 0,9% NaCl solution (≥99% glycerol, Sigma Aldrich, Darmstadt, Germany), 4) IGF-I solution-injected group: 100 ng/mL of IGF-I dissolved in 0,9% NaCl solution (human insulin-like growth factor-I, Sigma Aldrich, Darmstadt, Germany, (Kocamis et al., 1998), 5) GLY + IGF-I solution-injected group:10 nmol/mL of GLY + 100 ng/mL of IGF-I dissolved in 0,9% NaCl solution. The pH and osmolarity of the solutions to be injected were evaluated using a pH meter (W3B; Bel Engineering, Monza, Italy) and an osmometer (K-7400, Kanauer, Berlin, Germany) (Table 1).

On 17.5 d of incubation, all injected solutions were freshly prepared and kept in the incubator at 30 °C to avoid thermal shock to the embryo. The eggs of the injected groups were candled to identify the location of amnion after disinfecting with 2% iodinated alcohol in the region of needle insertion. A volume of 0.5 mL of each solution was injected into the amniotic fluid using a 1-mL disposable syringe and a 22-gauge needle. After injection, the hole in the shell was sealed with paraffin (Gonçalves, et al., 2013). After each injection, the needles were replaced. Although the control group was not injected, it was subjected to the same procedures as the in injected groups. After inoculation, the eggs were placed in bride sacs containing the identification of the experimental group (Pedroso, et al., 2006). The whole process was carried out in a sanitized room that was maintained at an average temperature of 30 °C. All eggs were held outside the incubator for less than 1 hour to complete the injection process, including the non-injected control group. Until hatch, all eggs were incubated according to the routine procedure. It is noteworthy that to ensure that the injection site was the amniotic fluid, 15embryonated test-eggs were injected with a water-soluble dye and then broken to verify the injected site.

2.3 Bird Housing

The hatching times were registered. At each examination period, the numbers of chicks hatched were counted and the hour registered. After 26 days of incubation, the non-hatched eggs were opened and the embryo mortality period was classified before and after injection by embryodiagnosis (Cobb, 2013). Hatchability was obtained by dividing the number of eggs that hatched with viable chicks by the number of fertile eggs determined on the 17.5 d of incubation.

Upon hatch, the chicks were housed separately, according to the experimental group, in 2.0 m long × 1.5 m wide boxes with floors covered with shavings, a tubular feeder and an

infant-type drinker, which was replaced 7 days later by the pendulum type; the boxes were in a screened masonry shed pre-heated with infrared lamps. Chicks were allowed free access to feed and water. When there were no more hatched eggs, 48 hours after the first bird had hatched, each chick birds were weighed, sexed and housed in mixed lots. The experimental design was a completely randomized design with a 2 × 2 + 1 (with or without GLY, with or without IGF-I, plus an additional treatment - non-injected eggs) factorial scheme, with five replicates of 5 birds each.

Continuous fluorescent illumination was maintained, and the temperature of the experimental room was monitored through two thermohygrometers (Simpla TH02, Asko, São Leopoldo, Brazil) placed in the shed at the height of the birds. The ingredients and nutrient levels of diets were formulated to meet the nutrient requirements (Rostagno, et al., 2011) (Table 2). The chicks were raised until 42 d of age. At d 7, 14, 21, 35 and 42, birds and feed were weighed, and feed intake was recorded by replicate to calculate the weight gain, feed intake and feed conversion.

2.4 Sample Collection

Upon hatch, randomly selected eight male birds per experimental group were individually weighed and euthanized by cervical dislocation. Blood samples were collected during bleeding. After collection, the blood was centrifuged at 1000 × *g* for 15 minutes (Sorvall TM ST16 Centrifuge, Thermo Fisher Scientific, Massachusetts, USA) to obtain plasma. The plasma was the stored at -80 °C for IGF-I, glucose and uric acid analysis.

From the same sample chicks, the weights of heart, liver, gizzard, yolk sac, breasts, thigh plus drumstick and small and large intestine were determined. The liver and breast were immersed in liquid nitrogen for a few seconds and stored at -80 °C for fructose-1.6-bisphosphato phosphatase (FBP) activity and hepatic and muscular glycogen determination.

The segments from the duodenum, jejunum and ileum were flushed with physiological saline and fixed in Bouin solution for 24 h. At 7 d of age, two male birds from each box were selected, according to the average weight of the box. These birds were euthanized following the same procedure described for hatch chicks.

At 42 d of age, two male birds with weights close to the box average were selected and slaughtered, feathered and eviscerated. The relative weight of carcass (without legs and abdominal fat), viscera, breast (skin and bone), wings and leg (thigh plus drumstick with skin and bone) were calculated in relation to live weight.

2.5. Biochemical Analyses

IGF-I levels were determined in plasma samples of chicks by an enzyme-linked immunosorbent assay (Human IGF-I ELISA kit, Sigma Aldrich, Darmstadt, Germany), according to the manufactures's instructions. Glucose and uric acid were quantified by an enzymatic colorimetric test (Liquiform Glucose, Liquiform Uric Acid, Labtest, Lagoa Santa, Brazil), following the manufacturer's recommendations.

For FBP activity, the liver (approximately 0.2 g) was homogenized in 1 mL of solution containing 0.1 M Tris-HCl (pH 7.5), 0.15 M KCl, 5 mM dithiothreitol and 5 mM $MgSO_4$. After tissue homogenization, the sample was centrifuged (20600 × *g* for 60 minutes at 4 °C) and the supernatant was collected according to the methodology proposed by Aoki, et al. (1999). The determination of the enzymatic activity was performed according to Ulm, et al. (1975).

The hepatic and muscular glycogen contents were determined according to Willems, et al. (2014). For this, liver and breast fragments (approximately 0.2 g) were homogenized in 7% perchloric acid solution. After complete homogenization of the tissue, the sample was

centrifuged (14000 × *g* for 15 minutes at 4 °C) and the supernatant was collected. Quantification was determined by color reagent based on iodine.

2.6. Intestinal Morphometry

Samples of the duodenum, jejunum and ileum with approximately 5 mm in length were subjected to routine histological procedures, being dehydrated in solutions with increasing concentrations of ethanol, cleared in xylene, embedded in paraffin, microtomized to 4.0 µm, arranged on silanized glass slides, dried at 37 °C overnight and stained with haematoxylin and eosin (Suvarna, et al., 2018). Two cuts of different regions were performed for each intestinal segment, of which scanned images were analysed using an Olympus microscope (CX31; Olympus, Tokyo, Japan) coupled to an Altra SC30 digital camera (Olympus) using the AxioVision program (Carl Zeiss, Oberkochen, Germany) at 100 x magnification for the duodenum, 200 x for the jejunum and ileum to birds at one day of age and 100 x for the different segments to birds seven days of age. Ten readings were taken per slide, measuring villus height and crypt depth. Villus height was measured from the tip of the villi to the villus crypt junction and crypt depth was defined as the depth of the invagination between adjacent villi. The average value was calculated per variable per chick. Later, villus height: crypt depth ratio was calculated (VH/CD).

2.7. Statistical Analyses

The hatchability data were submitted to a binomial model and the main effect was analyzed by alikelihood ratio test. The means were compared using a Pearson Chi-square test. The other data were analyzed to tests for normality (Shapiro–Wilk), homoscedasticity of variance (Breusch–Pagan) and independence of errors (Durbin–Watson); in the case of non-significance on these tests, an analysis of variance (ANOVA) was performed to determine the

main effects and the interaction between GLY and IGF-I. The statistical model was $Xij = \mu + \alpha i + \beta j + (a\beta)ij + eij$, where μ = overall mean, αi = GLY effect, βj = IGF-I effect, $(a\beta)ij$ = interaction between GLY and IGF-I, eij = error contribution with average 0 and variance $\sigma 2$. Significance was accepted at $P<0.05$. A Scott–Knott test was used to detect significant differences between means whenever an interaction between GLY and IGF-I was observed. Otherwise, a Dunnett's test was used to detect significant differences between means from the factorial scheme and the control (non-injected). For the non-standard ANOVA variables, the Box-Cox or Johnson data transformation options were used. The variables that did not meet the assumption of normality, even after data transformation, were submitted to non-parametric analysis and the means were compared by a Kruskal–Walis test. All statistical analyses were performed in the statistical programs Sisvar version 5.6 (DEX/UFLA, Lavras, Minas Gerais, Brazil) and Action version 3.4 (Estatcamp, São Carlos, São Paulo, Brazil).

Results

3.1. Hatch and Biochemical Parameters

Hatchability, hatch time and glycemia did not differ among *in ovo* injection treatments ($P>0.05$) (Table 3 and 4). The IGF-I increased ($P<0.01$) the concentrations of IGF-I in the plasma at hatch. Higher concentrations of plasma uric acid were observed ($P<0.05$) with injection of saline or GLY. There was no interaction ($P>0.05$) between GLY and IGF-I for hepatic and muscular glycogen. The injected with GLY or IGF-I had increased ($P=0.01$) concentrations of hepatic glycogen in comparison to those in control. Increased ($P=0.05$) muscular glycogen concentration was obtained with GLY. The injection of GLY, or IGF-I, or GLY + IGF-I resulted ($P<0.05$) in lower FBP activity, compared to the control groups (saline solution or non-injected).

3.2 Body Composition

At 1-d of age, a higher relative weight liver (P=0.05) was observed with injection of GLY, or IGF-I or GLY + IGF-I. Saline solution, GLY or IGF increased the relative weight of duodenum (P=0.02). IGF-I increased (P<0.05) the relative weight small intestine. There was no effect (P>0.05) on the other carcass characteristics evaluated at this age.

At 7-d of age, GLY reduced (P<0.01) the relative weight liver and IGF-I increased (P=0.02) the relative weight ileum (Table 5) There was no effect (P>0.05) on the other carcass characteristics evaluated at this age. There was also no effect (P>0.05) on carcass composition at 42 days of age.

3.3 Intestinal Morphometry

There was no effect (P>0.05) of the interaction between GLY and IGF-I on intestinal morphometry (Table 6). The GLY promoted greater (P <0.01) villus height the ileum in comparison to the control at 1 and 7 d of age. At 1 d of age, GLY also increased crypt depth in the ileum relative to the control group and IGF-I decreased villus height (P=0.03) in the jejunum.

3.4 Performance

The performance of the chicks during the first week of life did not differ (P>0.05) among *in ovo* injection treatments (Table 7). At 14 d, higher feed intake was observed (P=0.03) with injection of GLY, or IGF-I or GLY + IGF-I in comparison to those in control. Greater weight gain was observed (P<0.01) with IGF and better feed conversion was observed (P<0.05) with IGF-I or GLY + IGF-I

At 21 d of age higher feed intake (P≤0.01) with GLY was observed. Weight gain was higher (P<0.01) with GLY, IGF-I or GLY + IGF-I. GLY+IGF-I increased (P=<0.05) weight gain in comparison to those in control. A lower feed conversion (P=0.01) was observed with IGF-I or GLY + IGF-I. At 35 d of age, lower feed intake and greater weight gain were observed (P<0.01) with saline solution, GLY, IGF-I or GLY + IGF-I in comparison to those in control. There was no effect (P>0.05) on feed conversion among *in ovo* injection treatments. At 42 d of age, higher feed intake (P<0.01) were observed with saline solution, GLY, IGF-I, or GLY + IGF-I, in comparison to those in control. Greater weight gain was observed (P<0.01) with GLY or IGF-I or GLY + IGF-I.

Discussion

The *in ovo* feeding technique has produced promising results for the improvement of the development of birds before and after hatch. However, there is still no consensus on the best substrates, concentrations or the different combinations to be used. In the present study, injection with GLY and IGF-I produced satisfactory results. We observed that, compared to intact eggs, supplementation with GLY + IGF-I improved the weight gain of the birds at 21, 35 and 42 d of age, without significantly influencing the composition of the carcass at the different ages. Although these results are considered positive, the use of GLY or IGF-I was also very beneficial. IGF-I was the only substance that led to better performance of the birds in the second week of life. To date, there have been no reports that have associated an energetic compound, such as GLY, with a metabolism-regulating agent, such as IGF-I, in solutions for use in *in ovo* injection of broilers.

Reports in the literature suggest that IGF plays an important role in embryo growth and development (McMurtry, et al., 1997). Physiological concentrations of IGF are able to accelerate metabolism and stimulate the differentiation of embryonic myoblasts in birds

(Schmid, et al., 1983). This may explain why the highest weight gain was observed in birds from the second week of life when IGF was used in the present study. Kocamis et al. (1998) also observed that the injection of rh-IGF-I (100 ng/embryo) on day 1 and 4 of embryonic development increased the body weight of 42 d of age chickens. According to the authors, this result was due to the significantly increased growth of skeletal muscle during both the embryonic and post-hatch stages. In fact, the largest synthesis of DNA in primary cultures of satellite cells from chicken muscle has been associated with IGF (Duclos, et al., 1991), demonstrating that this substance plays an important role in tissue metabolism.

Like IGF-I, GLY also positively influenced the post-hatch performance of the birds. The greater weight gain observed at 14, 35 and 42 d of age may be related to the fact that GLY had been used by the embryos as a gluconeogenic substrate in the final stage of embryonic development, reducing the use of amino acids and increasing the contribution of these nutrients to protein synthesis, favoring the development of muscle fibers in the embryos (McMurtry, 1998; Lu, et al., 2007). Although IGF is considered an important stimulator of muscle protein synthesis, in the present study, little additional gain was observed when GLY + IGF-I was injected. This result may be related to the tissues' control of endogenous IGF release.

The availability of amino acids is one of the factors that stimulate plasma IGF concentration (Kita, et al., 2002). In this case, the likely increase in plasma IGF-I, resulting from GLY injection, may have stimulated the release of endogenous IGF-I by the embryo. Although the GLY in action did not present higher physiological IGF-I concentrations at hatch, the hypothesis that GLY may have some influence on IGF release is reinforced by the higher plasma IGF-I concentration in chicks from eggs injected with GLY + IGF-I, in relation to those injected with IGF-I alone. These differences may be related to the measurement technique used, in which only free IGF-I is quantified. In the body, the IGF concentration is

modulated by IGF-I binding proteins (IGFBPs). The formation of IGF-IGFBP complexes in serum can modify the bioavailable IGF-I pool by sequestering IGF-I or by decreasing IGF-I turnover (McQueeney and Dealy, 2001). In this case, the increase of endogenous IGF-I, stimulated by GLY supplement, may not be detected by the technique that was used. In turn, IGF-I can control the activity of IGFBPs by regulating their gene expression or by altering the activity of their proteases (Wetterau, et al., 1999). In this case, the presence of the exogenous IGF-I provided by the injection of GLY + IGF-I may have mitigated the effects of IGFBPs on free IGF-I. This would explain the higher plasma IGF-I concentration in chicks from eggs injected with GLY + IGF-I. In addition, the presence of exogenous IGF may have reduced the additional effect of endogenous IGF-I stimulated by GLY, since the increased IGF-I concentration may activate mechanisms that regulate the action of this hormone in tissues (Sjögren, et al., 1999).

GLY increased the relative weight of the liver in birds at one week of age. In nutrition research, liver weight represents an indirect way to determine the metabolic activity of the organ (Zaefarian, et al., 2019). The greater deposition of hepatic glycogen provided by the GLY and IGF-I solutions reinforces the hypothesis that these substances influence the metabolic activity of the liver. However, Neves et al. (2016) did not observe an effect of different concentrations of GLY on the relative weight of broiler livers, but they observed a greater development of the intestinal villi and greater hepatic glycerol kinase activity. In the present study, greater development of intestinal villi in the jejunum and ileum could also be observed after hatching and at 7 days. In this case, the probable increase of the intestinal villi, caused GLY solutions, may have increased the supply of nutrients to the liver in the first week of life and, therefore, may have stimulated the metabolism of this organ.

We also observed that IGF-I did not significantly influence the relative weight of organs in general. In ducks, an increase in the post-hatch morphological differentiation of the small

intestine has been reported on the 13th day of incubation after the inoculation of growth factors in the amniotic fluid (Wang, et al., 2012). According to the authors, IGF-1 plays an important role in cell proliferation and organ formation through different physiological forms. In the present study, the lower relative weight of the intestine observed in birds at 1 d of age, the increase of only the ileum in the first week of life and lack of improvements in the development of intestinal epithelium were not expected. The difference in the results obtained from different studies may be related to the species used or the time of the injection of the substances.

Regarding the evaluated biochemical parameters, the reduction of FBP activity in newly-hatched poultry from eggs inoculated with GLY and IGF-I, associated or not, suggests less gluconeogenic activity at the end of the hatching period (Chaekal, et al., 1983). This result may be associated with the effects of GLY and IGF-I on hepatic glycogen deposition moments before hatching, providing enough carbohydrates for the maintenance of glycemia during the hatch process. The lower concentration of uric acid observed in the newly-hatched birds from eggs inoculated with IGF indicates that this hormone plays an important role in the utilization of amino acids by the tissues, directing the amino acids for protein synthesis (Karcher, 2005) and reducing the contribution of these nutrients to the synthesis of glucose. In fact, Liu et al. (2011) showed that IGF-1 can affect duck muscle mass during the late hatch stages after *in ovo* injection of IGF-1 on the 12th day of incubation. According to Watford et al. (1981), gluconeogenesis depends on amino acids, lactate and glycerol. In this case, this important metabolic pathway began to use other gluconeogenic substrates instead of amino acids, reducing the production of uric acid in the liver. Although the biochemical results found in the present study suggest a higher amino acid use for protein synthesis, live weight at hatch was not affected by the different injected solutions. This result may be related to the time of the injection of the solutions (17.5^{h} d of incubation).

5. Conclusion

The positive results observed in the present study suggest that *in ovo* injection may be considered a viable technique to be applied in commercial hatcheries. Therefore, the use of solutions containing GLY or IGF-I, associated or not, can bring benefits to the performance of the birds, without harming egg hatchability. However, further research should be done to consider other broiler strains. In addition, the results of the present study suggest that both IGF-I and GLY play an important role in the embryo metabolism of broilers. In this case, molecular biology studies aimed at elucidating the mechanisms of action of these substances in the embryonic cells are necessary.

Disclosure statement

No potential conflict of interest was reported by the authors.

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1623 **Table 1.** pH and osmolarity of the used solutions.

Solution	pH	Osmolarity (mOsm)
0.9% saline solution	7.00	324
10 nmol/mL of glycerol in 0.9%saline solution	7.39	325
100 ng/mL of IGF-I in 0.9%saline solution	7.56	330
10 nmol/mL of glycerol + 100 ng/mL of IGF-I in 0.9%saline solution	7.52	329

Table 2. Centesimal composition and calculated nutritional levels of the experimental diets used in the different stages of post-hatch development of broilers.

Ingredient	1-7 d	8-21 d	22-35 d	36- 40 d
Corn	55.489	59.645	62.377	67.093
Soybean meal 45%	38.162	34.664	31.437	27.218
Soy oil	2.055	2.013	2.963	2.787
Dicalcium phosphate	1.907	1.508	1.273	1.070
Calcitic limestone	0.914	0.924	0.865	0.771
Salt	0.507	0.482	0.457	0.444
DL-Methionine (99%)	0.359	0.287	0.256	0.240
L-Lysine (78%)	0.289	0.219	0.196	0.237
L-Threonine (99%)	0.114	0.064	0.043	0.053
Salinomycin	0.050	0.050	0.050	0.000
Vitamin supplement [1]	0.050	0.040	0.030	0.020
Mineral supplement [2]	0.050	0.050	0.050	0.050
Choline chloride (60%)	0.050	0.050	0.000	0.020
Avilamycin	0.005	0.005	0.005	0.000
TOTAL	100.00	100.00	100.00	100.00
Calculated nutritional composition				
Metabolizable energy, kcal/kg	2950	3000	3100	3150
Crude protein, %	22.20	20.80	19.50	18.00
Calcium, %	0.920	0.819	0.732	0.638
Available phosphorus, %	0.470	0.391	0.342	0.298
Digestible lysine, %	1.310	1.174	1.078	1.010
Digestible methionine + Cystine, %	0.944	0.846	0.787	0.737
Digestible threonine, %	0.852	0.763	0.701	0.656
Sodium, %	0.220	0.210	0.200	0.195

1. Supply per kg of product: 5.000.000 IU of vitamin A; 1.850.000 IU of vitamin D3; 4.500 IU of vitamin E; 918 mg of vitamin K3; 2.000 mg of vitamin B2; 250 mg of vitamin B6; 6,500 mcg of vitamin B12; 145.4 mg of folic acid; 12.9 g of niacin; 5,931 mg of pantothenic acid and 480 mg of selenium.

2. Supply per kg of product: 67.5 g of manganese; 50.4 g of iron; 43.2 g of zinc; 7.0 g of cupper and 1,464 mg of iodine.

1632 **Table 3.** Hatchability and incubation time of non-injected eggs and injected eggs with
1633 glycerol (GLY, 10 nmol/mL) or IGF-I (100 ng/mL) or GLY + IGF-I. (n = 80)

Variable	Non-injected	Saline solution	GLY	IGF-I	GLY + IGF-I	P=
Hatchability (%)	79.71	86.11	87.69	90.48	85.71	0.49
Incubation time (h)	514	513	513	508	509	0.14

Table 4. Biochemical parameters and carcass composition of 1-d of age broiler chicks from non-injected eggs and injected eggs with glycerol (GLY, 10 nmol/mL), IGF-I (100 ng/mL) or GLY + IGF-I. (n = 8)

Variable	Treatment					Main effects GLY		Main effects IGF-I		Probabilities				SEM
	Non-injected	Saline solution	GLY	IGF-I	GLY + IGF-I	0	10	0	100	GLY	IGF-I	GLY x IGF-I	Non-injected	
IGF-I (ng/mL)[2]	1.91c	1.98c	1.94c	2.53b	2.98a	2.26	2.46	1.96	2.76	0.73	<0.01	0.05	<0.01	-
Uric acid (mg/dL)[1]	4.38b	6.79a	5.23a	4.56b	4.96b	5.68	5.10	6.01	4.76	0.65	0.01	0.06	0.05	-
Blood glucose (mg/dL)	206.3	199.2	191.5	205.9	203.1	202.6	197.3	195.4	204.5	0.40	0.15	0.69	0.37	6.21
Hepatic glycogen (mg/g)	0.70	0.89	1.04*	1.02*	0.96	0.96	1.00	0.97	0.99	0.66	0.76	0.27	0.01	0.09
Muscle glycogen (mg/g)[2]	0.60	0.53	0.62	0.57	0.62	0.55	0.62	0.58	0.60	0.05	0.96	0.65	0.95	-
FBP ($\times 10^{-6}$)	10.87 a	9.57 a	6.93 b	5.33b	7.26 b	7.45	7.10	8.25	6.30	0.78	0.13	0.05	0.02	1.27
Live weight (g)	48.13	50.68	48.14	49.86	49.39	50.27	48.77	49.41	49.63	0.11	0.82	0.27	0.19	0.92
Breast (%)	4.85	4.66	4.68	4.59	4.73	4.63	4.71	4.67	4.66	0.51	0.91	0.62	0.17	0.11
Thigh + drumstick (%)[2]	6.80	6.45	6.61	6.50	6.48	6.48	6.55	6.53	6.49	0.62	0.72	0.33	0.10	-
Heart (%)	0.70	0.75	0.79	0.74	0.76	0.75	0.78	0.77	0.75	0.29	0.38	0.66	0.09	0.03
Liver (%)	2.32 b	2.26 b	2.45 a	2.43 a	2.43 a	2.35	2.44	2.36	2.43	0.08	0.17	0.05	0.23	0.05
Gizzard (%)	4.58	4.25	4.45	4.31	4.25	4.28	4.35	4.35	4.28	0.54	0.50	0.22	0.14	0.11
Duodenum (%)	0.58 b	0.63 a	0.69 a	0.68 a	0.55 b	0.66	0.62	0.66	0.62	0.36	0.19	0.02	0.14	0.04
Jejunum (%)[2]	0.72	0.78	0.84	0.8	0.72	0.79	0.78	0.81	0.76	0.83	0.31	0.21	0.14	-
Ileum (%)	0.6	0.65	0.66	0.65	0.6	0.65	0.63	0.66	0.63	0.54	0.46	0.46	0.36	0.04
Small intestine (%)	1.98	2.11	2.34	2.12	1.91	2.12	2.13	2.23	2.02	0.88	0.04	0.13	0.21	0.10
Large intestine (%)[2]	1.55	1.62	2.43	1.70	1.65	1.66	2.04	2.03	1.68	0.89	0.89	0.80	0.15	-
Yolk residue (%)	15.6	14.95	15.41	15.63	15.57	15.29	15.49	15.18	15.60	0.70	0.41	0.61	0.72	0.51

FBP - Fructose 1,6 Bisphosphato phosphatase; SEM: standard error of the mean
[1] Transformed data (transformation option: Box-Cox)
[2] Transformed data (transformation option: Johnson)
[a,b] Means assigned different letters within a factor of analysis (GLY and IGF-I and their interactions) are significantly different by Scott-Knott test (P<0.05).
* Mean differs from the control (non-injeced) by a Dunnett´s test (P<0.05)

Table 5. Body composition (% in relation to live weight) of different aged broilers from non-injected eggs and injected eggs with glycerol (GLY, 10 nmol/mL), IGF-I (100 ng/mL) or GLY + IGF-I. (n = 5)

Variable	Treatment					Main effects GLY		Main effects IGF-I		Probabilities				SEM
	Non-injected	Saline solution	GLY	IGF-I	GLY + IGF-I	0	10	0	100	GLY	IGF-I	GLY x IGF-I	Non-injected	
7 d of age														
Slaughter weight (g)	181	175	191	181	173	178	182	183	177	-	-	-	-	-
Breast (%)	16.5	16.4	16.9	17.6	16.1	17.0	16.5	16.7	16.8	0.36	0.77	0.16	0.65	0.40
Thigh + drumstick (%)	8.10	7.75	7.84	8.37	8.08	8.06	7.96	7.80	8.23	0.75	0.17	0.52	0.79	0.24
Heart (%)	0.98	0.84	0.85	0.93	0.80	0.89	0.85	0.85	0.93	0.18	0.59	0.12	0.12	0.03
Liver (%)	3.84	4.01	3.34	3.64	3.55	3.83	3.45	3.68	3.60	<0.01	0.42	0.11	0.08	0.08
Gizzard (%)	6.58	6.32	6.52	6.32	7.19	6.32	6.86	6.42	6.76	0.10	0.29	0.29	0.98	0.24
Duodenum (%)	1.66	1.93	1.69	1.79	1.95	1.86	1.82	1.81	1.87	0.65	0.53	0.14	0.10	0.07
Jejunum (%)	2.63	2.73	2.54	2.55	3.01	2.64	2.78	2.64	2.78	0.30	0.26	0.12	0.59	0.10
Ileum (%)	2.16	2.42	1.93	2.22	2.73	2.32	2.33	2.18	2.48	0.95	0.02	0.10	0.25	0.10
Small intestine (%)	6.46	7.09	6.16	6.56	7.37	6.83	6.77	6.63	6.97	0.79	0.14	0.10	0.19	0.17
Large intestine (%)	2.08	1.99	1.88	2.31	2.11	2.15	2.00	1.94	2.21	0.36	0.11	0.80	0.97	0.13
42 d of age														
Slaughter weight	3546	3502	3701	3676	3532	3589	3617	3601	3604	-	-	-	-	-
Carcass (%)[1]	76.5	77.8	78.0	77.9	77.9	77.8	77.9	77.9	77.9	0.64	0.76	0.50	0.70	-
Breast (%)	32.1	31.6	31.6	31.3	31.8	31.4	31.7	31.6	31.6	0.47	0.71	0.92	0.70	0.62
Thigh + drumstick (%)	20.2	20.8	20.9	20.7	20.4	20.8	20.7	20.9	20.6	0.96	0.35	0.44	0.20	0.33
Wings (%)	7.21	7.14	7.23	7.30	7.35	7.22	7.29	7.19	7.33	0.73	0.34	0.96	0.84	0.13
Heart (%)	0.46	0.47	0.48	0.50	0.50	0.49	0.49	0.48	0.50	0.81	0.37	0.70	0.25	0.02
Liver (%)	1.64	1.56	1.59	1.64	1.52	1.60	1.56	1.58	1.58	0.77	0.83	0.26	0.59	0.06
Gizzard (%)	1.45	1.47	1.36	1.44	1.39	1.46	1.38	1.42	1.42	0.69	0.73	0.91	0.87	0.08
Duodenum (%)	0.42	0.41	0.39	0.39	0.39	0.40	0.39	0.40	0.39	0.95	0.50	0.98	0.45	0.02
Jejunum (%)	0.84	0.84	0.80	0.86	0.79	0.85	0.80	0.82	0.83	0.34	0.86	0.91	0.70	0.05
Ileum (%)	0.65	0.71	0.73	0.78	0.70	0.75	0.72	0.72	0.74	0.24	0.32	0.59	0.17	0.03
Small intestine (%)	1.92	1.96	1.92	2.02	1.87	1.99	1.90	1.94	1.95	0.24	0.70	0.80	0.97	0.08
Large intestine (%)	0.58	0.65	0.62	0.65	0.67	0.65	0.65	0.64	0.66	0.89	0.58	0.59	0.22	0.04
Abdominal fat (%)[1]	1.30	1.35	1.16	1.24	1.06	1.30	1.11	1.26	1.15	0.52	0.26	0.73	0.57	-

SEM: standard error of the mean

[1] Transformed data (transformation option: Johnson)

Table 6. Intestinal epithelium morphometry of different aged broilers from non-injected eggs and injected eggs with glycerol (GLY, 10 nmol/mL), IGF-I (100 ng/mL) or GLY + IGF-I. (n = 8 and 5)

Variable	Treatment					Main effects GLY		Main effects IGF-I		Probabilities				SEM
	Non-injected	Saline solution	GLY	IGF-I	GLY + IGF-I	0	10	0	100	GLY	IGF-I	GLY x IGF-I	Non-injected	
1 d of age														
Duodenum														
VH	415	449	461	446	440	447	450	455	443	0.91	0.66	0.75	0.25	9.39
CD	52	59	61	51	52	55	57	60	52	0.70	0.07	0.98	0.45	1.62
VH/CD	7.5	7.9	8.1	9.0	8.1	8.4	8.1	8.0	8.5	0.37	0.19	0.53	0.33	0.20
Jejunum														
VH	271	270	281	249	238	260	259	275	244	0.97	0.03	0.43	0.47	4.96
CD	39	45	42	40	44	42	43	43	42	0.69	0.53	0.24	0.18	0.94
VH/CD	6.5	6.2	6.4	6.4	5.5	6.3	6.0	6.3	6.0	0.06	0.37	0.17	0.30	0.13
Ileum														
VH	216	242	282*	264	246	253	264	262	255	0.47	0.63	0.06	0.01	5.08
CD	47	59	64*	53	56	56	60	62	54	0.33	0.14	0.85	0.05	1.68
VH/CD	4.8	4.2	4.5	5.1	4.4	4.7	4.5	4.4	4.8	0.55	0.17	0.12	0.53	0.11
7 d of age														
Duodenum														
VH	768	862	805	757	747	809	776	833	752	0.53	0.14	0.65	0.68	23.65
CD	125	134	131	130	144	132	137	132	137	0.49	0.54	0.27	0.25	3.28
VH/CD	6.3	5.6	5.5	5.8	5.3	5.7	5.4	5.6	5.5	0.67	0.97	0.72	0.32	0.31
Jejunum														
VH	539	642	623	603	641	622	632	633	622	0.80	0.78	0.45	0.06	16.61
CD	147	159	157	160	170	159	164	158	165	0.71	0.61	0.65	0.33	5.91
VH/CD	4	4.1	4.0	4.0	3.8	4.1	3.9	4.0	3.9	0.58	0.72	0.97	0.94	0.18
Ileum														
VH	425	479	513*	409	498	444	505	496	454	0.01	0.07	0.22	0.05	9.78
CD	122	133	127	114	134	123	130	130	124	0.08	0.15	0.06	0.31	1.71
VH/CD	3.4	3.6	3.9	3.6	3.7	3.6	3.8	3.7	3.6	0.10	0.54	0.83	0.06	0.05

VH: villus height (µm); CD: crypt depth (µm); VH/CD (µm/µm)

SEM: standard error of the mean

* Mean differs from the control (non-injected) by a Dunnett´s test (P<0.05)

Table 7. Performance of different aged broilers from non-injected eggs and injected eggs with glycerol (GLY. 10 nmol/mL). IGF-I (100 ng/mL) or GLY + IGF-I. (n = 5)

Variable	Treatment					Main effects GLY		Main effects IGF-I		Probabilities				SEM
	Non-injected	Saline solution	GLY	IGF-I	GLY + IGF-I	0	10	0	100	GLY	IGF-I	GLY x IGF-I	Non-injected	
1 to 7 d														
Feed intake (g)	155	151	162	164	158	158	160	157	161	0.42	0.21	0.12	0.31	2.45
Weight gain (g)	140	135	139	142	138	139	138	137	140	0.85	0.19	0.18	0.46	1.76
Feed conversion	1.15	1.14	1.16	1.13	1.15	1.14	1.16	1.15	1.14	0.04	0.33	0.81	0.89	0.01
1 to 14 d														
Feed intake (g)	679 b	673 b	694 a	699 a	689 a	686	692	683	694	0.39	0.13	0.03	0.19	5.22
Weight gain (g)	523 c	528 c	540 b	555 a	537 b	542	538	534	546	0.27	<0.01	<0.01	<0.01	2.41
Feed conversion	1.29 a	1.29 a	1.30 a	1.26 b	1.26 b	1.26	1.28	1.28	1.26	0.12	0.05	0.05	0.04	0.01
1 to 21 d														
Feed intake (g)	1444 b	1426 b	1486 a	1446 b	1457 b	1436	1471	1456	1451	<0.01	0.61	0.01	0.31	6.76
Weight gain (g)	1050	1037	1069	1063	1082*	1050	1076	1053	1073	<0.01	0.01	0.30	0.05	5.00
Feed conversion	1.37	1.37	1.38	1.36	1.35	1.36	1.37	1.38	1.35	0.88	0.01	0.17	0.54	0.01
1 to 35 d														
Feed intake (g)	3790	3970	3948*	3958*	3943*	3964	3945	3959	3951	0.63	0.83	0.93	<0.01	29.24
Weight gain (g)	2373	2425	2539*	2487*	2525*	2493	2532	2519	2506	0.03	0.44	0.93	<0.01	13.27
Feed conversion	1.61	1.64	1.58	1.59	1.58	1.61	1.58	1.61	1.58	0.17	0.31	0.44	0.54	0.02
1 to 42 d														
Feed intake (g)	5197	5554*	5439*	5418*	5384*	5486	5411	5497	5401	0.18	0.09	0.46	<0.01	42.75
Weight gain (g)	3088	3181	3254*	3243*	3208*	3212	3231	3218	3226	0.48	0.77	0.15	<0.01	20.80
Feed conversion	1.74	1.74	1.69	1.71	1.68	1.73	1.69	1.71	1.70	0.19	0.68	0.74	0.38	0.02

SEM: standard error of the mean

[a.b] Means assigned different letters within a factor of analysis (GLY and IGF-I and their interactions) are significantly different by a Scott–Knott test (P<0.05).

* Mean differs from the control (non-injected) by a Dunnett's test (P<0.05).

CONCLUSÕES DA TESE

Os resultados positivos observados no presente estudo sugerem que a injeção *in ovo* pode ser considerada uma técnica viável a ser aplicada em incubatórios comerciais. Portanto, o uso de soluções contendo 100 ng/mL de IGF-I pode ser utilizado para aumentar o número de ovos eclodidos e o uso de 10 nmol/mL de glicerol, associado ou não, a soluções de 100 ng de IGF-I/embrião pode trazer benefícios para o desempenho das aves, sem prejudicar a eclodibilidade dos ovos.

No entanto, mais pesquisas devem ser feitas para considerar outras linhagens de frangos de corte. Além disso, os resultados do presente estudo sugerem que tanto o IGF-I quanto o glicerol desempenham um papel importante no metabolismo embrionário de frangos de corte. Nesse caso, são necessários estudos de biologia molecular com o objetivo de elucidar os mecanismos de ação dessas substâncias nas células embrionárias.

ANEXO

UNIVERSIDADE FEDERAL DE LAVRAS
PRÓ-REITORIA DE PESQUISA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS
Cx.P.3037 - Lavras - MG - 37200-000 - (35) 3829-5182 cea@nintec.ufla.br

CERTIFICADO

Certificamos que o protocolo nº 049/14, relativo ao projeto intitulado Nutrição in ovo: Adição de glicerol e fator de crescimento semelhante à insulina (IGF-I) em ovos embrionados de frangos de corte, que tem como responsável Márcio Gilberto Zangeronimo está de acordo com os princípios éticos da experimentação animal, adotados pela Comissão de Ética no Uso de Animais (Comissões Permanentes/PRP-UFLA), tendo sido aprovado na reunião de 25/09/2014.

Início do projeto:01/11/2014 - Término do projeto:30/06/2015.
Espécie: Ave - Quantidade de animais: 1360.

CERTIFICATE

We hereby certify that the Protocol nº 049/14, related to the project entitled "Nutrition in ovo: Addition of glycerol and insulin-like growth factor (IGF-I) in embryonated eggs of broilers", under the supervision of Márcio Gilberto Zangeronimo, is in agreement with the Ethics Principles in Animal Experimentation, adopted by the Institutional Animal Care and Use Committee (Standing Committees/PRP-UFLA), and was approved in September 25, 2014.

Project's beginning:01/11/2014 - Project's end:30/06/2015.
Especies: Ave - Number of animals: 1360.

Lavras, 25 de setembro de 2014

Prof. Gabriela Rodrigues Sampaio
Presidente da Comissão de Ética no Uso de Animais-CEUA

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ATESTADO

Conforme solicitação do pesquisador **Márcio Gilberto Zangeronimo**, a CEUA aprova a prorrogação do prazo do projeto intitulado "Nutrição in ovo: Adição de glicerol e fator de crescimento semelhante à insulina (IGF-I) em ovos embrionados de frangos de corte", **protocolo nº 049/14,** até março de 2019.

Lavras, 26 de novembro de 2015.

Profª. Gabriela Rodrigues Sampaio
Presidente da Comissão de Ética no Uso de Animais CEUA